The Protective Role of Melatonin in Small Liver Graft Transplantation

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1. Summary

Liver transplantation is the only curative therapy for end-stage liver diseases. It has developed from a medical miracle into a routine surgical procedure and has rescued numerous patient's lives. During the past few decades, the brilliant work of transplant surgeons and scientists has tackled various surgical and immunological issues which used to be the main obstacle of liver transplantation. However, until now, we still cannot provide sufficient organs for the patients in need. Strategies such as living donor liver transplantation (LDLT) have been implemented to increase the donor liver pool. In addition, the extreme shortage of organs have triggered the surgeons to transplant smaller partial grafts and thereby expanding the donor pool. This method has expanded the availability of donor livers but is hindered by the development of small for size syndrome (SFSS). Melatonin (MLT) is a hormone that shows a variety of functions using both physiological or pharmacological doses. The exogenous high dose of MLT is particularly important in protecting organs after ischemia and regulating various bodily functions. These features of MLT have inspired us to investigate its potential role in small for size (SFS) liver transplantation.

In the first part of the study, we developed different animal models aiming to verify the hypothesis whether MLT protects small liver graft through reducing ischemia reperfusion injury (IRI) and promoting liver graft regeneration. With the model of ischemia reperfusion plus partial hepatectomy (I/R+PH), MLT exhibits an exceptional beneficial effect in terms of reducing the injury and enhancing the regeneration of remnant liver. When the remnant liver volume is further reduced to a lethal size, MLT significantly improved the survival rate of animals compared with the control group. In a 30% arterialized partial liver transplantation model which we have defined as a small for size liver transplantation (SFS-LT) model, MLT increased the animal survival rate accompanied by a dramatic upregulation of IL-6 mRNA expression and IL-6 downstream pathway activation in the liver graft. Regarding the mechanism, we confirmed that the beneficial effect of MLT is MLT receptor independent, and by using IL-6 knockout mice and gp130 inhibitor, we demonstrated that IL-6 signaling pathway is indispensable for the beneficial effect of MLT in our study. In addition, the positive effect of MLT can be partially attributed to its role of improving the hepatic microcirculation, which was displayed as better sinusoidal reperfusion and less leukocyte adhesion.

During liver transplantation, the donor organ requires speedy retrieval and implantation to reduce warm and cold ischemia time, therefore, this surgery can be taken place at any unpredicted time of the day depending on the availability of donors. This uncertainty of operating time arises a question that whether the circadian rhythm of human body plays a role in hepatic IRI and the outcome of liver transplantation recipients. In the second part of

the thesis, we applied mice hepatic IRI model to answer this question. Surgeries were performed at different selected time points of the day, compared with sham group, an obvious shift of circadian gene expression was observed 6 hours after each surgical time point. Nighttime (ZT18) operations showed significantly attenuated hepatic injury compared with daytime (ZT7) surgeries 6 hours post hepatic I/R. Mice with 6 hours fasting before the onset of ischemia eliminated the beneficial effect of nighttime surgeries, however, it showed a protective role in daytime operations.

In conclusion, this thesis focused on two clinically relevant studies: We demonstrated that exogenous MLT improves the outcome of small liver graft transplantation through reducing IRI and promoting graft regeneration. This effect depends on the activation of IL-6 signaling pathway and the protection of graft microcirculation. This study supports the clinical application of SFS liver grafts in living donor liver transplantation (LDLT) and will eventually increase the donor pool. In addition, we observed that circadian rhythm and pre-operative fasting influence the short-term outcome after hepatic IRI. This study provides an evidence that circadian rhythm should be taken into consideration for better perioperative care of liver transplant recipients.

2. Zusammenfassung

Die Lebertransplantation ist die einzige kurative Behandlung für terminale Lebererkrankungen. Die Lebertransplantation ist in den letzten Jahrzehnten zu einer Routineoperation geworden und hat viele Leben gerettet. Die Einführung in den klinischen Alltag wurde durch intensive Forschung von Chirurgen und Wissenschaftlern möglich gemacht, die chirurgische und immunologische Probleme zu lösen hatten. Nichtsdestotrotz, gibt es immer noch zu wenig Organe um allen Patienten eine Leber zu garantieren. Verschiedenste Strategien zur Verbesserung des Organmangels wurden evaluiert, so zum Beispiel die Leber-Lebend-Spende. Zusätzlich wurde versucht kleine Teil-Leberstücke zu implantieren, sodass auch hier dem Organmangel entgegengewirkt werden konnte. Die Teil-Lebertransplantation birgt jedoch Gefahren für den Empfänger, der eine Leberdysfunktion mit erheblichen Komplikationen erleiden kann, das sogenannte Small-for-Size Syndrom (SFSS).

Melatonin (MLT) ist ein Hormon mit komplexen physiologischen Funktionen. Nach Gabe einer pharmakologischen Dosis von MLT zeigte sich ein Schutz vor Ischämie-Reperfusionsschaden in verschiedenen Organen, unter anderem auch nach einer Leberresektion. Deshalb wollten wir prüfen, ob die Wirkung von MLT auch bei komplexeren Eingriffen, z.B. der Teillebertransplantation einen Schutz bringt.

Im ersten Teil unserer Studien entwickelten wir verschiedene Tiermodelle, um die Hypothese zu testen, dass MLT einen Schutz bei der Teilleberresektion und dem danach folgenden Reperfusionsschaden aufbaut, sowie die Leberregenration fördert. Als erstes wurde ein kombiniertes Resektiosn/Leberschadenmodell etabliert (IR+PH). Bei diesem hat die perioperative Gabe von MLT eine ausserordentliche, schützende Wirkung indem es den Schaden reduziert und die Regeneration fördert. Bei einer sehr kleinen Restleber, nach Resektion von 80%, hat sich die MLT Gabe positiv auf das Überleben ausgewirkt. In einem nächsten Schritt wurden 30% Leber transplantiert, was normalerweise zu einem SFSS mitfatalem Ausgang führt. Bei zusätzlicher Gabe von MLT konnte auch hier das Überleben signifikant erhöht werden. Gleichzeitig wurde eine starke Erhöhung von IL-6 mRNA beobachtet. IL-6 fördert die Regeneration und wir konnten zeigen, dass in genetisch IL-6 defizienten Mäusen, MLT nicht mehr wirksam ist. In zusätzlichen Experimenten, mittels Applikation eines Inhibitors des IL-6/gp130 Protein Komplexes, konnte gezeigt werden, dass der protektive Effekt von MLT von diesem Signaltransduktionsweges abhängig ist. MLT hatte auch einen positiven Effekt auf die Mikrozirkulation im regenerierenden Gewebe.

Das zweite Projekt stellte sich die Frage, ob die Tageszeit der Transplantation eine Rolle auf die postoperativen Komplikationen hat. Im Gegensatz zu elektiven Operationen, werden Transplantationen zu jeder Tages- und Nachtzeit durchgeführt, um das Organ vor langer Ischämie zu schützen. Die meisten Organismen unterliegen einem zirkadianen Rhythmus, der metabolische Aktivitäten kontrolliert. Zu diesem Zweck wurden Experimente mit Ischämie und Reperfusion an Mauslebern durchgeführt. Verschieden Zeitpunkte wurden untersucht, wobei sich stellvertretend zwei für die Analyse am besten eigneten: ZT7 (Tag) und ZT18 (Nacht). Nach einer Reperfusion von 6 und 24 Stunden wurden die Tiere untersucht. Erstaunlicherweise war der Leberschaden geringer, wenn die Mäuse, im Gegensatz zu ZT7, bei ZT18 (aktive Phase) operieret wurden. Um den Einfluss der Nahrungsaufnahme zu testen, wurden Mäuse für 6 Stunden gefastet. In diesem Fall kehrte sich das Bild und die Mäuse, die während der Tageszeit (ZT7, Ruhephase) operierte wurden, waren besser vor Reperfusionsschaden geschützt. Eine zusätzliche physiologische Gabe von MLT hatte keine positive Wirkung.

Unsere Experimente habe sich auf klinisch relevante Probleme fokussiert. Wir konnten zeigen, dass MLT eine protektive Wirkung auf den Leberschaden nach verschiedenen Lebereingriffen hatte. Diese Wirkung konnte nur entfaltet werden, wenn die Signalkaskade IL-6/gp130 intakt war. Damit könnte man die Lebend-Leber-Transplantation sicherer gestalten und allenfalls den Pool an Spenderorganen erhöhen. Zusätzlich haben wir beobachtet, dass der zirkadiane Rhythmus und die präoperative Nahrungsaufnahme den Ausgang der Lebertransplantation wesentlich beeinflussen.

3. Introduction

3.1 Liver

The liver, located in the upper right quadrant of the abdomen, is the second largest organ and the biggest gland in the human body. It has two distinct sources of blood supply, including oxygenated blood from the hepatic artery and nutrient-rich blood from the portal vein [1]. Based on the unique anatomical distribution of vessels, the liver can be divided into two main lobes which are further divided into eight segments [2]. The segments are made up of thousands of lobules which are considered the building blocks of the liver. The liver lobule, roughly a hexagonal arrangement of six hepatocytes is characterised by a terminal branch of hepatic vein in the center, while terminal branches of the hepatic artery, portal vein and bile duct converge on each of the six corners. This structure is called the portal triad, and is the basic functional unit of the liver [3]. The liver cell populations are recognized as two groups: parenchymal and non-parenchymal cells. 60-70 % of total liver cells are parenchymal hepatocytes while 30-40% of the liver cells are non-parenchymal cells (NPCs). NPCs comprise numerous cell types, including cholangiocytes, Kupffer cells, hepatic stellate cells and sinusoidal epithelial cells [4]. Reflecting its unique and sophisticated anatomical and histological features, the liver bears a variety of vital functions (Figure 1), to support the body with its metabolic and energy demands and to deal with foreign toxic challenges. If one of the hepatic functions is impaired, it may result in deterioration of other body functions and, eventually lead to liver disease.

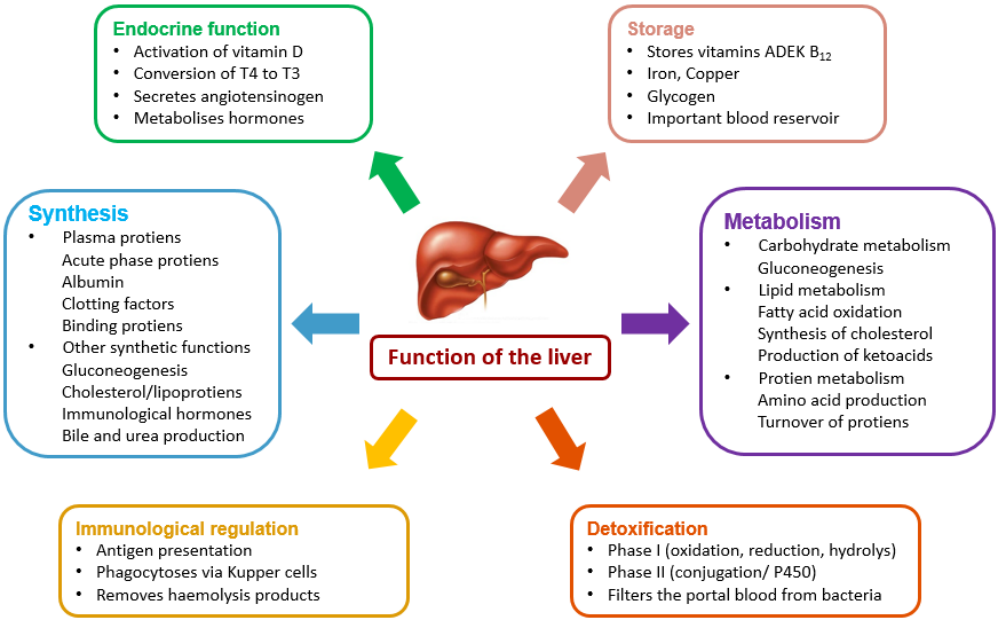

Figure 1. The functions of the liver [5-7].

3.2 Liver transplantation

Various internal (congenital and genetic reasons) and external (virus, alcohol) factors can cause the malfunction of the liver. If the pathogenesis progresses rapidly or the patient cannot receive effective treatment, the disease may develop to a decompensated phase, or so-called end-stage liver disease (ESLD), which is no longer reversible [8].

Thanks to one of the greatest medical breakthroughs of the last century, patients with ESLD can now be successfully treated by liver transplantation. Since its first successful performance in 1967 by Dr. Thomas Stazl [9], liver transplantation has become the only curative treatment for ESLD and has already rescued thousands of lives [10]. Decompensated cirrhosis has been the most prevalent indication for liver transplantation, although the etiology differs among different parts of the world, from different types of hepatitis to alcoholic liver cirrhosis [11]. Patients with acute liver failure is considered as a highly-urgent status for liver transplantation and should be transferred immediately to a transplant center [12]. The cases of liver transplantation for malignancy have dramatically increased in the past 15 years in the US, from 7.7% in 2002 to 22.4% in 2012, [13]. In addition, some other rare diseases such as primary sclerosing cholangitis (PSC), autoimmune hepatitis (AIH) and inherited metabolic liver diseases can also be treated with liver transplantation and excellent results have been achieved [14, 15]. As for children, biliary atresia is the most common indication for liver transplantation [16].

In recent years, with the improvement of surgical techniques and peri-operative management, the 5-year overall survival rate of liver transplantation has reached more than 70% [17]. However, unlike the patient with end-stage renal disease who can still lead a relatively normal life for an extensive period with the application of dialysis [18], liver disease and in particular liver failure, hepatic loss of function cannot be bridged. The complicated circulation system and multiple functions of the liver have so far prevented the development of an artificial liver supporting system to replace hepatic function . Therefore, patients with ESLD have no long-term perspective unless they undergo liver transplantation. Furthermore, patients with liver malignancy require speedy resection or transplantation to avoid tumor progression [19, 20]. Nevertheless, the number of liver transplantation is steadily increasing year by year, the number of patients who are de-listed from the waiting list or die while waiting for a new liver is also increasing [21]. This is caused by a continuously widening gap between the growing number of patients and the supply of organs. Thus, the main challenge for both transplant surgeons and patients is the shortage of donor livers [22].

3.3 Strategies to increase the donor pool

The worsening situation of donor liver shortage has triggered investigations into different new methods to increase the liver donor pool (Table 1) [23-28]. New techniques such as 3-D printing and tissue engineering are also considered aiming to produce new organs [24]. These techniques can generate the shape of an artificial organ; however, organ function cannot be fully reproduced [25]. Xenotransplantation was considered one of the first methods to increase the donor pool. Unfortunately, these early attempts in humans in the 1960s resulted in a poor survival rate and raised severe ethical concerns. Therefore, no application using xenotransplantation has reached clinical practice. This technique will not be approved in the near future until a solution has been found to overcome the strong immune rejection and the infection of animal derived diseases [26, 29]. Both artificial organ and xenotransplantation might become promising techniques for organ replacement in the future, but they are currently not contributing to change the situation of organ shortage.

Strategies	Pros	Cons	Examples
Man-made organs	• Expand organ availability • No organ allocation issues	• Technical issues • Functional recovery	• Tissue engineering • 3-D printing
Xenotransplantation	• Easy harvesting • Expand organ availability	• Immune rejection • Animal derived infectious diseases	• Pigs • Monkeys
Marginal organs	• Expand organ availability	• Poor organ quality • Organ optimization measures required • Recurrence of original diseases	• DCD • Fatty liver • Old liver • Diseased liver
Partial liver transplantation	• Short cold ischemic time • Decrease recipients' waiting time	• Concerns of donor safety • Small for size syndrome • Surgical complications	• Split liver transplantation • Living donor liver transplantation
Expand organ donation	• Increase citizen's awareness of organ donation • Increase donor source	• Restricted by religious beliefs in Eastern countries	• Opt out policies
Refine organ matching system	• Better organ allocation • Individualized medicine	• Complexity of system development • Not all the situations can be considered	• Optimize allocation system

Table 1. Pros and cons for strategies to increase the liver donor source.

Currently, the application of marginal livers and living donor liver transplantation (LDLT) are the most widely used strategies to expand the donor pool [30]. According to different cultural background, the popularity of the

two measures varies from eastern and western parts of the world. Although there is not a standard definition for marginal livers, we in general consider the donor livers as marginal if they meet one of the following criteria: aged donor (donor age>65), adverse past medical history (HBV, HCV infection), concerns of pre-existing liver damage or disease (donor after cardiac death, fatty liver) [31]. Not until recent years when newly invented techniques were applied to optimize the quality of these donors, they were thought to be inferior and related to poor outcome of the recipients. Machine perfusion offers an *ex vivo* dynamic preservation for donor organs. There is an ongoing debate on the optimal temperature for perfusion, but this does not stop the increased interest in using this technique to preserve marginal livers in order to improve the quality and viability of grafts [27, 32]. While the western world is searching optimal measures to expand the source and improve the quality of marginal organs, the eastern world is more focusing on the use of living donors [33]. LDLT accounts for more than 80% of all the liver transplantations in Asia compared with less than 5% in the US and Europe [34]. This phenomenon results from cultural and religious traditions in Asian countries which restrict organ donation after death [35].

3.4 Living donor liver transplantation

Due to the unique regenerative capability of the liver, LDLT provides an excellent option to increase the donor pool. Since its first performance in 1989, LDLT has obtained a remarkable breakthrough in the field of liver transplantation [36]. In recent years, LDLT has not only achieved equal outcomes as deceased donor liver transplantation (DDLT) but also significantly extended the liver donor pool [37]. Especially in Asia, this technique accounts for the majority of liver transplantation activities. During the last two decades, although the surgery and peri-operative management of LDLT have greatly progressed, some controversial issues remained. Among them, the question whether one should use the left or the right lobe has been an unresolved debate. The appropriate size of the liver graft, sufficient to sustain the recipient and is safe for the donor, is still debated. The initial approaches for adult LDLT included the use of the right lobe as graft, which accounts for two thirds of the liver volume. However, right lobe hepatectomy is related to higher rates of donor morbidity and mortality [38, 39]. Donor safety has the most important priority in LDLT from an ethical perspective, a medical procedure should not be performed that does not guarantee a healthy donor's safety [40]. Therefore, with the technical refinements and innovative management strategies, several experienced centers start using left lobe for LDLT [41]. After years of practice, left lobe LDLT has resulted in excellent outcomes for both donor and recipients, most importantly, it expanded the liver donor pool. Originally, most centers performing LDLT would consider a safe graft when its weight is greater than 40% of the recipient's standard liver volume or more than 0.8% of the recipient's body

weight [42, 43]. With better patient selection and improved surgical skills, the safe limit of minimum graft weight to standard liver volume ratio has been reduced to 35% and 0.6% of graft to recipient's body weight [44, 45].The latter liver grafts are considered small liver graft. In general, there are currently two reasons that encourage the experienced surgeons to use small liver grafts: Firstly, using small liver grafts could simplify the surgical process and improve the safety of the donor. Secondly, statistics confirmed that every 5% reduction of graft weight to standard liver volume ratio results in an almost doubling of the LDLT cases. Thus, application of small liver graft could potentially increase the availability of donor livers and shorten the recipient's time on the waiting list [46]. Nevertheless, every coin has two sides; if the small liver graft does not meet the functional and metabolic demand of the recipient, the recipient may develop Small-for-Size (SFS) Syndrome (SFSS) which is life threatening. Thus, the advantage of using small liver graft cannot be extended beyond certain limits to avoid the risk for SFSS.

3.5 SFSS and its preventive strategies

The term SFSS has been used to describe a clinical syndrome characterized by the presence of hyperbilirubinemia, prolonged coagulopathy, encephalopathy and ascites in the absence of other surgical or drug caused liver failure within the first week after liver transplantation [47]. The major mechanism of SFSS is the shear stress caused by over-perfusion of portal inflow through a small liver graft. An arterial hypoperfusion and outflow obstruction also contribute to the development of SFSS [48]. However, SFSS is not only related to a reduced graft size, it has been reported that SFSS can occur even with right lobe LDLT [49]. Therefore, several other factors such as graft quality, donor age, surgical skills and recipient's clinical status also result in the development of SFSS [48]. In particular, liver regeneration and functional recovery after LDLT are essential for the outcomes of recipients and the combination of hepatic injury and regenerative failure are believed to be important factors of SFSS [50].

Liver regeneration after LDLT has long been a research focus although very limited advancements have been achieved so far. Our research group and others, using standardized mouse models have demonstrated that mice can tolerate 70% hepatectomy (30% remnant volume) very well. with a complete volume recovery within 7-10 days. However, when mice receive a volume of 30% liver volume through arterialized liver transplantation, most mice died in 2-4 days, probably due to liver failure and SFSS [51]. The same remnant liver volume in these two models resulted in completely different outcome, it indicates that the knowledge of liver regeneration in

hepatectomy model cannot be fully reproduced in partial liver transplantation model. In liver transplantation settings, the harvesting and implantation of the donor liver inevitably cause extra insult to the graft, which is known as cold and warm ischemic reperfusion injury (IRI) [52]. Oxidative stress, intracellular calcium overload and cellular immune activation are critically involved in the pathogenesis of IRI [53]. IRI not only contributes to poor recipient's outcomes after liver transplantation, but also makes the liver graft more vulnerable to other kind of damage. This can explain why SFSS could occur in right lobe LDLT, although the liver volume is apparently sufficient for the recipient. The functional volume is actually inadequate to support the recipient's daily metabolism needs in combination with the regenerative process. For small liver graft, if we don't take effective measures to reduce those injuries, a primary regeneration defect may occur and eventually lead to SFSS.

Current strategies in preventing or treating SFSS include three major measures: portal inflow modulation, liver outflow modulation and pharmacological treatment (Table 2) [48, 54]. Since endothelial shear stress and portal hyperperfusion are considered as the main mechanisms of SFSS, different methods to reduce portal hyperperfusion have been investigated. Early trials used portosystemic shunting techniques to reduce the portal pressure [55], however, this technique is related to several unwanted consequences caused by secondary portal flow insufficiency, such as encephalopathy and graft atrophy [56]. Therefore, portosystemic shunting cannot offer a permanent solution for SFSS. Splenic artery modulation is another strategy to reduce portal inflow. It can be performed during the operation as prophylaxis for SFSS, or in the early post-operative period when SFSS is diagnosed [57]. For high-risk patients who receive small liver grafts, portal pressure and flow monitoring should be conducted routinely, if the portal pressure is higher than 20 mmHg or portal flow higher than 20 ml/min/100g, portal flow modulation should be performed [58, 59]. Due to the risk of hemorrhagic and septic complications, splenectomy is not recommended to reduce portal pressure [60]. Thus, splenic artery embolization and ligation are the most common methods to modulate portal inflow. In addition, outflow obstruction is one of the risk factors for developing SFSS, especially in the case of right lobe LDLT. Therefore, the appropriate drainage of anterior segments, and whether the middle hepatic vein should be included in the donor liver, should be seriously evaluated before the operation [48, 61]. Apart from surgical startegies, pharmacological treatment is another option to prevent or treat SFSS. The main mechanism of pharmacological treatment is to decrease portal shear stress and to enhance regeneration of the liver. Several vascular regulators such as prostaglandin E1, nitric oxide and endothelin receptor antagonist have been investigated in animals and promising results have been reported [62, 63]. Our group has shown that pentoxifylline has the potential to promote liver regeneration after major hepatectomy and small liver graft transplantation in both animal models and human trials [64, 65]. However, due to the lack of

multi-center randomized controlled trials in humans, the application of different pharmacological treatment in SFSS is still limited.

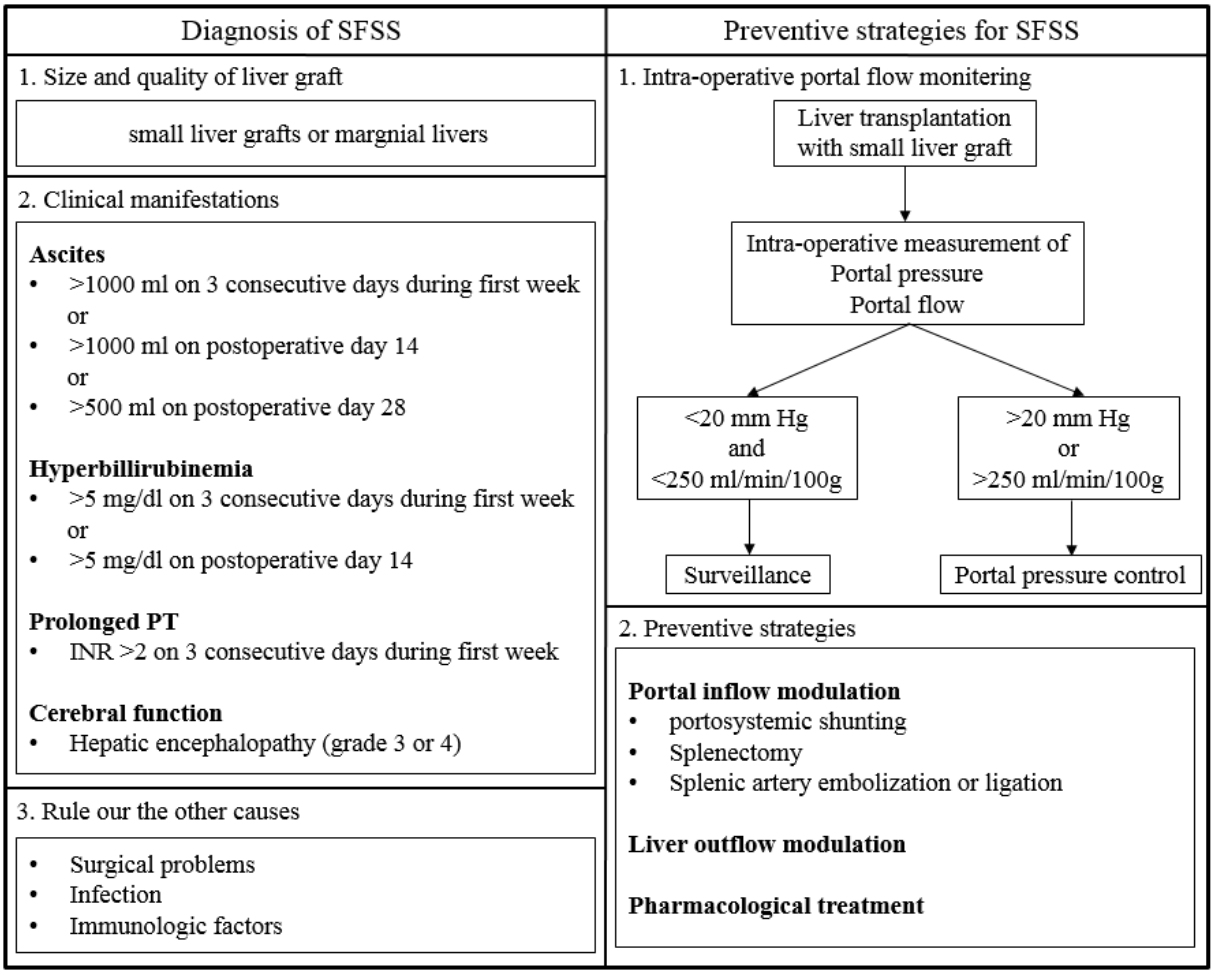

Table 2. Current opinion in the diagnosis and management of SFSS after LDLT, adopted from [48, 54, 59].

With the development of surgical techniques and peri-operative care, SFSS is now better prevented. However, SFSS is still the leading obstacle for LDLT because many problems remain unresolved, partially due to the multifactorial nature of SFSS. Current treatment cannot take all the risk factors of SFSS into consideration. Most of the treatments focus on portal flow modulation, but the fact that IRI and other kinds of graft injury also contribute to the development of SFSS are still neglected. Therefore, new techniques and novel drugs which have the potential to prevent or treat SFSS, need to be investigated.

3.6 Melatonin: a versatile hormone in human body

Since it was first purified and characterized in 1958, a large number of studies concerning the biosynthesis, metabolism, physiology and pathology of melatonin (MLT) have accumulated. In recent years, Consistent efforts have uncovered a wide range of physical functions of MLT and it is considered as one of the most versatile hormones in the human body [66]. MLT has been identified in both plants and animals. In vertebrates, melatonin is mainly secreted by the pineal gland, however, the secretion process is also observed in other parts of the body, including the retina, bone marrow, platelets, the gastrointestinal (GI) tract, skin and lymphocytes [67-72]. The synthesis of MLT in the pineal gland is dependent on the daily light/dark cycle. During darkness, the absence of light is registered by the retina and further projected to the suprachiasmatic nucleus (SCN) through the retinohypothalamic tract. Then, the SCN signals the pineal gland to produce MLT [73, 74]. The precursor of MLT is tryptophan. During synthesis of MLT, tryptophan is taken up from the blood and converted into serotonin via 5-hydroxytryptophan. Serotonin is further acetylated to N-acetylserotonin with the catalysis of arylakylamine N-acetyltransferase (AA-NAT), this is regarded as the rate-limiting step of the whole process. Afterward, MLT is synthesized from N-acetylserotonin by hydroxyindole O-methyltransferase (Figure 2) [75]. The production of MLT shows a typical circadian rhythm, with a high secretion level during night and a low level during the day, this characteristic is independent of whether the species are diurnal or nocturnal. Thus, MLT is also known as the hormone of darkness [76]. The synthesis of MLT from peripheral organs also follow a similar circadian periodicity [77]. The metabolism of circulating MLT is mainly performed in the liver through cytochrome P 450 system, it is principally metabolized to 6-hydroxymelatonin (6-HMEL) and further conjugated with sulfate and excreted into urine [66]. Several actions of MLT are mediated by its membrane receptors MT1 and MT2. Both of them belong to the superfamily of G-protein coupled receptors. In mammals, melatonin receptors have been found in the brain and several peripheral organs. However, the density and location of MLT receptors expression varies between different organs and species [78]. These receptors contribute to several physiological actions, among those, circadian regulation is one of the most important and well-known functions of MLT. In spite of some activities through its receptors, MLT is a lipophilic hormone which can diffuse through biological membranes of cells without binding to its receptors. This unique characteristic enables MLT to exert a series of functions independent of its receptors. With the accumulated evidence, it is now generally accepted that endogenous MLT takes its action in a receptor dependent way, while high dose exogenous MLT is more likely to function with a receptor independent manner (Table 2) [79-81].

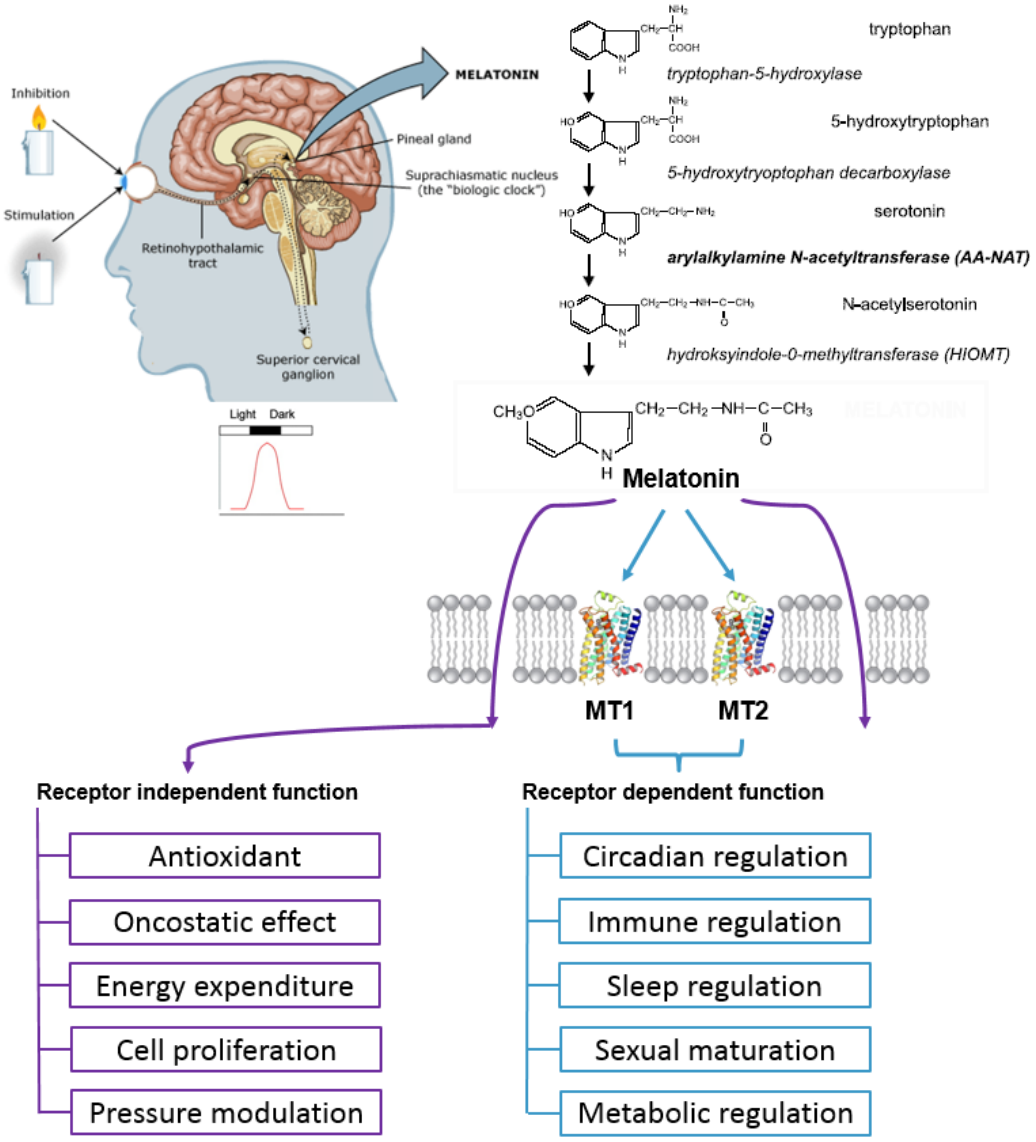

Figure 2. Biosynthesis and physiological function of MLT, adopted from [66, 79, 80, 81].

Among all its known functions, circadian regulation and anti-oxidative effect are the most well-known and investigated topics of MLT. Both have already been translated into clinical practice. The role of MLT in sleep regulation is one of its receptor dependent circadian regulatory actions. The American Academy of Sleep Medicine recommends that MLT can be used in jet lag, sleep disorder and other situations like circadian disturbance induced poor sleep quality [82]. The effect of MLT in circadian regulation can be attributed to its action in stabilizing circadian genes and stimulating adenosine signaling [83, 84], with MT2 being the major player [85]. Since the anti-oxidative effect of MLT was unveiled, numerous studies on this feature have been triggered. Evidence shows that melatonin is considerably more efficient than the majority of any other antioxidants [86]. The anti-oxidative effect of MLT results from both direct and indirect manners. Directly, MLT acts as a scavenger by

neutralizing reactive nitrogen species (RNS) and reactive oxygen species (ROS) [87]. Indirectly, MLT stimulates the production of anti-oxidative enzymes such as glutathione (GSH) and superoxide dismutases (SOD). It also induces the downregulation of pro-oxidant enzymes such as nitric oxide synthase (NOS) and lipoxygenases [88]. Besides, melatonin could preserve mitochondrial function by reducing electron leakage and preventing ATP depletion [89]. The anti-oxidative effect of MLT is particularly important in light of its protective role in warm IRI after organ transplantation.

3.7 Melatonin in transplantation

Organ transplantation is a complex procedure and many factors can induce failure of the surgery. IRI is an unavoidable injury of all transplanted organs and a leading cause of graft failure. The anti-oxidative property of MLT renders it protective against IRI. Many studies have confirmed that MLT reduces liver injury after warm IRI [90]. Apart from the anti-oxidative effect of MLT, several other molecular pathways including heme oxygenase-1 (HO-1), toll-like receptor (TLR), c-Jun N-terminal kinase (JNK) are actively involved in this process [91-93]. In addition to warm IRI, transplanted organs usually undergo cold ischemic injury during the cold preservation period between organ retrieval and implantation. Organs with poor quality such as marginal organs are more vulnerable to this kind of injury. Adding MLT to the perfusion and preservation solution protects both steatotic and nonsteatotic liver grafts against cold IRI [94, 95]. The liver form elderly donors is another type of marginal organ. Kireev et al [96] observed that older animals suffered much worse from hepatic damage compared with young animals after IRI. Melatonin administration reduced such damage in both groups with a more significant reduction in older rats. Besides the benefits of MLT in cold and warm IRI, it has also been shown that melatonin could prevent immune rejection after heart transplantation by inhibiting the proliferation of lymphocytes and reduce immune rejection after pancreatic islet transplantation through decreasing the proportion of Th1 cells and elevating the percentage of IL-10 producing cells [97, 98]. Biliary complications are the most common complications following liver transplantation. They occur more frequently in aging livers and remain an intractable problem after LDLT [99]. Besides surgical and anatomical reasons, chronic bile duct hyperplasia is a chronic graft dysfunction and the main cause of biliary complications [100]. One study showed that MLT could inhibit cholangiocyte hyperplasia via MT1 [101].It indicates that MLT may have the potential to prevent biliary complications after liver transplantation. What's more, as portal hyperperfusion is one of the initial reasons in the development of SFSS after LDLT, reducing portal pressure is an important target for preventing SFSS. The effect of MLT to control blood pressure makes it a good candidate for pharmacological

treatment of SFSS [102]. After transplantation, microcirculation is vital for hepatic recovery and liver regeneration [103, 104], improved microcirculation has been found in different organs with the administration of MLT [105, 106]. These findings have strengthened the hypothesis that MLT is beneficial in preventing SFSS.

The application of high dose exogenous MLT application has raised the concern of safety issues. Early pharmacological studies tried to induce mortality of mice with increasing doses up to the highest possible dose of 800 mg/kg, given its solubility. Even at this does the IC50 could not be reached. [107], demonstrating that MLT can be safely used with high doses. In the clinic, one study in Heidelberg injected 50 mg/kg MLT to patients undergoing extended hepatectomy. All the patients tolerated MLT well, showed better liver function and shorter hospital stay compared with non-treated patients, [108]. The value of MLT has also been demonstrated in patient's peri-operative care. It significantly reduces post-operative pain and pre-operative anxiety when given at a high dose [109]. Taken together, MLT may improve the recipient's outcome through improving graft quality in different aspects following liver transplantation. Although the application of high dose MLT has appears to be safe in patients after surgery, its safety in recipients after liver transplantation still needs to be precisely evaluated.

3.8 Circadian rhythm and the liver

Circadian rhythm is a biological process generated from a transcription-translational feedback loop. The suprachiasmatic nucleus (SCN), located at the hypothalamus, is the major oscillator of circadian rhythm and coordinates clocks in the peripheral tissues [110]. Light is the predominant factor in entraining the central circadian cycle, while food can entrain the peripheral clocks independent of light [111]. A number of circadian genes are crucial in synchronizing the circadian rhythm into a 24-hour light/dark cycle [112]. Circadian locomotor output cycles kaput (Clock), brain and muscle-Arntlike 1 (Bmal1), Period 1, 2, 3 (Per1-3) and cryptochrome 1-2 (Cry1-2) are considered the main circadian genes. BMAL1 and CLOCK form a transcription heterodimer complex and promote the expression of negative loop components such as Pers and Crys, then the dimerization of the protein products of Per and Cry genes translocate into the nucleus in order to inhibit CLOCK:BMAL1 mediated transcription [113]. Genetic and environmental factors can disrupt the expression of circadian genes and lead to physiological disorder and disease [114]. The liver bears the most circadian genes and a wide variety of processes in the liver, including metabolism, nutrient uptake and detoxification are under circadian control [115]. In recent years, various liver diseases have been found to be circadian rhythm related (Figure 3) [110, 113], for

example, the clock mutation results in more increased lipid accumulation and fatty liver disease [116]. PER2 protein is important in protecting hepatic fibrosis and cirrhosis because more severe liver fibrosis and activation of hepatic stellate cells are observed in mPer2 knockout mice [117]. A study of human patients with colon or rectal cancer revealed that the gene expression of Bmal1 and Per1 in the abdomen is associated with the cancer metastasis to the liver [118]. Another study in mice showed that the dysfunction of circadian rhythm increased the incidence of cancer [119].

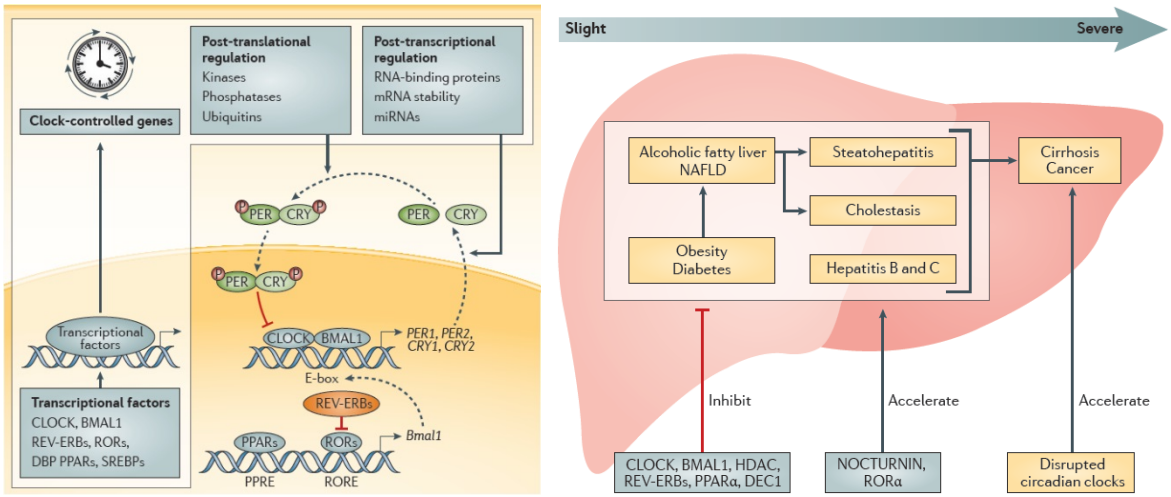

Figure 3. Basic mechanisms of circadian rhythm and the relations to liver diseases, adopted from [110].

Regarding IRI, people who have an irregular life style or work during nighttime have a higher incidence of developing cardiovascular diseases, besides, heart attack is more likely to happen at nighttime or early morning [120]. It has been demonstrated that the size of heart infarction is significantly larger when heart IRI was induced at nighttime with Per2 being a key factor contributing to the damage [121]. This observation reveals that circadian rhythm plays may affect IRI. Surgery is one of the causes for a disrupted circadian rhythm. Unlike routine surgeries, liver transplantation does not allow the surgeons to decide the timing of operation, because the availability of donor livers is not predictable. In order to reduce the cold ischemic time and keep the vitality of the livers, surgeries have to be performed as soon as the organs arrive, which means liver transplantation surgeries are performed any time of the day. To investigate whether the timing of liver transplantation influences the outcome of recipients, a study reported that the patients who were operated at night had longer time of operation and higher risk of early death than those patients who were operated during the day [122]. Although this study cannot exclude the fact that the poor outcome is associated with the less accurate surgical and anesthetic

skills at night and further studies are needed to investigate this observation further [123]. Still it provides the concept that circadian rhythm does play a role in liver transplantation surgeries. What's more, food is the second important factor for circadian regulation apart from light. It is particularly essential for the coordination of the peripheral clock [111]. Fasting is routinely required for patients before the operation, however, in patients undergoing liver transplantation, fasting is not as reliably organized due to the unpredictability of liver graft arrival. In addition, the length of fasting and the starting time of fasting could also affect the outcome of transplantation [124, 125]. Therefore, to fully understand and improve peri-operative care and the outcome of liver transplantation recipients, the impact of circadian rhythm and food intake should not be ignored.

4. Aim of the project

As described in the introduction, the deteriorating situation of organ shortage has become a major obstacle of liver transplantation and directly results in an increased death of patients on the waiting list. LDLT offers a promising solution to increase the donor pool, but the application of LDLT is restricted due to donor safety and development of SFSS. The multifunctional property of MLT makes it a potential therapeutic agent in various physiological disorders; these functions of MLT also benefit the transplanted livers through acting on different risk factors. However, the role of MLT in graft protection and liver regeneration after SFS liver graft transplantation has never been elucidated.

In the first part of our study, we developed different mouse models aiming to elucidate whether MLT could reverse SFS liver graft failure through reducing hepatic IRI and promoting liver regeneration. Furthermore, the underlying mechanisms are also investigated.

Circadian rhythm is involved in a variety of physiological activities. More attention has been paid in recent years concerning the impact of circadian rhythm in peri-operative care. In particular, the unpredictable timing of liver transplantation makes it necessary to understand the effect of the circadian rhythm in the recipients. Hepatic warm IRI is a leading risk factor for worsening recipient's outcome after liver transplantation. Cardiac research have unveiled the importance of circadian genes in heart IRI, whether this is reflected in other organs such as the liver is still unknown. Understanding the impact of circadian rhythm in hepatic IRI is essential in better scheduling the time of liver transplantation.

In the second part of our study, we used a mouse hepatic IRI model aiming to answer the question whether circadian rhythm and pre-operative fasting affect the outcome of hepatic IRI.

5. Manuscript A

Title:

Exogenous Melatonin Augments Graft Regeneration via Activating IL-6/gp130-Stat3 Signaling in Small-for-Size Liver Transplant

Short title:

Melatonin augments small liver graft regeneration

Author(s):

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Abbreviations:

Abbreviations used in this paper: *ALT, alanine aminotransferase; AST, Aspartate aminotransferase; DMSO, dimethyl sulfoxide; eNOS, endothelial nitric oxide synthase; gp130, glycoprotein 130; HGF, hepatocyte growth factor; HMGB1, High-Mobility-Group-Protein B1; HO-1, heme oxygenase-1; IL-6, interleukin-6; IL-6R, Interleukin-6 receptor; I/R+exPH, 60 min 20% hepatic ischemia plus 80% hepatectomy; I/R+PH, 60 min 30% hepatic ischemia plus 70% hepatectomy; iNOS, inducible nitric oxide synthase;JNK, c-Jun N-terminal kinase; LDLT, living donor liver transplantation; MLT, melatonin; NO, nitric oxide; PCNA, proliferating cell nuclear antigen; pH3, phospho-histone 3; rIL-6, recombinant interleukin-6; SFS-LT, small-for-size liver graft*

transplantation; SFSS, small-for-size syndrome; STAT3, signal transducer and activator of transcription 3; TLR, toll-like receptor; TNF-α, tumor necrosis factor alpha.

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Disclosures:

The authors have nothing to disclose

Contribution:

This is the main project during my PhD, I have performed most of the animal surgeries and several biological analysis. I am strongly involved in the design of the whole project and the analysis of experimental data. I drafted this manuscript.

Exogenous Melatonin Augments Graft Regeneration via Activating IL-6/gp130-Stat3 Signaling in Small-for-Size Liver Transplant

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Abstract

Background and aim: Living donor liver transplantation (LDLT) extends the liver donor pool, however, liver failure caused by small for size (SFS) syndrome (SFSS) restrict the application of LDLT. Exogenous melatonin (MLT) has shown protective effects on hepatic ischemia reperfusion injury (IRI), whereas its impact on graft regeneration after liver transplantation is unknown. We investigated the role of MLT on graft regeneration and IRI in a mouse model of SFS liver transplantation. **Methods:** We established three mouse models: (I) I/R+PH group: 60 min liver ischemia plus 2/3 hepatectomy, which mimics partial liver transplant; (II) I/R+exPH group: 60 min liver ischemia plus extended hepatectomy that simulates SFS liver transplant; (III) SFS-LT group: Arterialized 30% liver transplant in mice. Each group was subdivided into MLT or vehicle treated sub-groups. Hepatic injury, cytokine release, liver regeneration, animal survival rate were measured and monitored. The activation of IL-6/gp130-Stat3 pathway was assessed. IL-6-/- mice and SC144 (gp130 inhibitor) were applied for signaling blockage. **Results:** MLT reduced hepatic injury, elevated IL-6 and TNF-α level and augmented hepatocyte proliferation in I/R+PH mice. The improved regeneration by MLT was eliminated when I/R+PH was performed in IL-6-/- mice, but can be restored by recombinant IL-6 (rIL-6) injection. MLT significantly improved hepatic microcirculation and the mouse survival in I/R+exPH mice compared with controls. In SFS-LT mice, MLT activated IL-6/gp130-State3 signaling, promoted graft regeneration and raised recipient's survival. These protective effects were cancelled by SC144 treatment. **Conclusion:** MLT rescues SFSS by ameliorating liver IRI and augmenting SFS graft regeneration through activating IL-6/gp130-Stat3 signaling in mouse model of SFS liver transplantation.

Key words : Small for size liver transplantation; Melatonin; Small for size syndrome; Liver regneration.

Introduction

End stage liver disease, characterizing an irreversible liver failure, is one of the leading causes of death. Liver transplantation remains the sole curative therapy for end-stage liver disease. This therapeutic strategy has achieved over 70% 5-year overall recipient's survival rate, mainly due to improved surgical techniques, new immunosuppressive agents and optimized management of perioperative care [1]. Due to organ shortage, the gap between the growing number of patients in the waiting list and the supply of organs is continuously widening, resulting in many patient's death on the waiting list before receiving an available organ. The liver has an inherent regenerative capacity which can restore its original volume rapidly after major tissue loss or damage [2]. This unique characteristic makes hepatectomy and partial liver transplantation possible. Therefore, living donor liver transplantation (LDLT) was established and developed since 1990s [3].

As a promising technique to increase the number of donor organs, LDLT has been widely applied, especially in Asian countries where religious beliefs restrict organ donation after death [4]. In LDLT settings, the appropriate size of liver graft, which is sufficient to cure the recipient and safe to sustain live donor, is essential for a successful LDLT. Some transplant centers prefer to use relatively small liver graft because it can not only ensure the safety of donors, but also potentially increase the availability of donor livers [5]. However, If a small liver graft is too small to meet the functional demands, e.g. less than 40% of standard liver volume or 0.8 graft/body ratio, the recipient may develop a so-called small-for-size syndrome (SFSS) that can be life threatening [6]. Liver graft ischemic/reperfusion injury (IRI) has been known to impair remnant liver regeneration after LDLT [7, 8]. Such liver fail to regenerate is the major cause of SFSS. Supporting liver graft regeneration is the key to rescue SFSS [9]. Therefore, promoting regeneration and ameliorating IRI of the liver graft are important to prevent SFSS.

Melatonin (MLT) is a hormone mainly synthesized in the pineal gland. It is regarded as a versatile hormone in the human body since it acts on different organs and participates in a variety of body functions [10]. Endogenous MLT is a well-known regulator of circadian rhythm, while pharmacological doses of MLT show notable anti-oxidative properties, resulting in protection from IRI in various organs [11-13].

Warm and cold IRI worsen the quality of liver grafts and survival of recipients in liver transplantation. Some research reports have confirmed that MLT reduces liver damage after warm IRI. This beneficial effect is not only attributed to the anti-oxidative property of MLT, but also relies on several molecular pathways, such as heme oxygenase-1 (HO-1), toll-like receptor (TLR), c-Jun N-terminal kinase (JNK) [14-16]. Moreover, MLT has

been shown to protect both steatotic and nonsteatotic liver grafts from cold IRI [17]. In this study, we aim to explore the effect of MLT on liver regeneration and IRI in SFS liver transplantation.

Materials and methods

Animals

Male wild-type C57BL/6 mice were purchased from Charles River. IL-6 knockout (IL-$6^{-/-}$) mice with C57BL/6 background were obtained from Jackson laboratory. Animals were kept under a temperature-controlled environment with strict 12-hour light/dark cycle, a standard laboratory diet was given to all the animals. All experimental protocols were approved by Cantonal Veterinary office of Zurich and were performed according to guidelines of Animal Care Committee of University of Zurich. All surgeries were performed in the morning.

Animal experiments

To mimic partial liver transplantation, a 30% ischemic reperfusion injury combined with 70% hepatectomy (I/R+PH) was performed. A longitudinal midline incision was performed and the liver was freed from its ligaments under isoflurane inhalation anesthesia. The right lobe and caudate lobe, which account for about 30% of the liver volume, were clamped by vessel clamps for 60 min to induce warm ischemia. At the end of ischemia, the rest of the lobes (left and median lobes) were resected and reperfusion was achieved by removing the clamps. Then, an ischemic reperfusion injury combined with extended hepatectomy model (I/R+exPH), also regarded as a lethal model was used, in this model. Only the right lobe was clamped during ischemia and remained after reperfusion. Finally, 30% arterialized small-for-size liver transplantation (SFS-LT) in mice was performed as previously described [18].

MLT (sigma, M5250) was first dissolved in ethanol and further diluted with saline. A dose-effect experiment was performed in I/R+PH model with different dose of MLT (0.08, 0.4, 2, 10 mg/kg). All experiments were subsequently performed using the effective dose of MLT at 10 mg/kg, which was given 15 min before operation and immediately after reperfusion in I/R+PH and I/R+exPH models. For the SFSS-LT model, 20 μg/ml MLT was mixed with saline to perfuse the donor liver before harvesting. Additional 10 mg/kg MLT was given to the recipient 15 min before operation and immediately after the anhepatic phase. Vehicle was given to the animals in the control group. Luzindole (Tocris Bioscience, 877) was used to investigate whether the effect of MLT is receptor dependent. It was dissolved in 5% dimethyl sulfoxide (DMSO) and diluted in saline, then 5 mg/kg luzindole was injected 30 min prior to surgery through a peritoneal route. Recombinant Mouse IL-6 (R&D, 406-

ML) was given to IL-6$^{-/-}$ mice (20 ng/g, i.p.) 30 min before the operation. To block gp130, SC 144 (Tocris Bioscience, 4963) was dissolved in DMSO and further diluted with saline as described before [19], 10 mg/kg SC144 was given to mice 30 min before surgery, while control mice were injected with vehicle.

Mice were sacrificed at different time points after the operation. Blood samples were obtained from the infra-hepatic vena cava for serum biochemical analysis. Liver tissues were harvested and either frozen in liquid nitrogen immediately or fixed in 4% formaldehyde.

Serum transaminase

Blood samples obtained from the inferior vena cava were centrifuged (6000 rpm, 3 min) and supernatant was harvested. Aspartate aminotransferase (AST), alanine aminotransferase (ALT) were measured using a serum multiple biochemical analyzer (Dri-Chem 4000i, Fujifilm).

Enzyme Linked Immunosorbent Assay

Serum level of HMGB1 was measured by ELISA (Shino Test). IL-6 DuoSet ELISA kit (R&D systems) was used to measure serum levels of IL-6.

RT-PCR

Total RNA was extracted from 50 mg of liver tissue using TRIzol reagent (Invitrogen). ThermoScript reverse-transcription PCR system (invitrogen) was used for cDNA generation. TaqMan gene expression assay for IL-6 (Mm00446190_m1), TNF-a (Mm00443258_m1), HGF (Mm01135193_m1) and 18S rRNA internal control (TaqMan ribosomal RNA control reagents) were measured with ABI Prism 7000 Sequence Detector System (PE Applied Biosystems, Rotkreuz CH). Fold induction of mRNA expression was shown.

Western blotting

Liver tissue was homogenized in Radioimmunoprecipitation assay (RIPA) buffer containing a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Bradford protein assay (BioRad, Hercules, CA, USA) was used to determine protein concentrations. Aliquots corresponding to 60 μg proteins were separated by SDS–PAGE electrophoresis. Immunoreactive bands were detected by the V3 Western Workflow System (BioRad, Hercules, CA, USA), according to the manufacturer's protocols. The primary antibodies Anti-CD130 (gp130) antibody (Abcam, 202850), Anti-STAT3 (phospho Y705) antibody (Abcam 76315) and b-tubulin antibody (Cell signaling, 2128S) were incubated overnight at 4 ºC.

Histological assessment

Liver specimens were fixed in 4% PBS-buffered formalin and embedded in paraffin for histological analysis. Hematoxylin-eosin (H&E) staining was performed according to established protocols. Anti-PCNA antibody (Abcam, 29), anti-pH3 antibody (Abcam, 92628), anti-HMGB1 antibody (Abcam, 18256) and anti-gamma H2A.X antibody (Abcam, 2893) were used as primary antibodies. Secondary detection was performed by anti-rabbit IgG, HRP-linked antibody (Cell signaling, 7074S) and the iView DAB kit (Ventana Medical Systems). Positive cells for PCNA and pH3 were assessed in 10 random visual fields by manual counting.

Intravital Fluorescence Microscopy

Mice were prepared 3 hour after IR/exPH surgery as described [20] on an intravital fluorescence microscopy (AxioScope. A1; Zeiss, Feldbach, Switzerland). Microscopic images were captured by a charge coupled device camera (AxioCam HSm; Zeiss) using ×10 (N-Achroplan 10x/0,25, Zeiss) and ×20 (W N-Achroplan 20x/0,5; Zeiss) objectives in AxioVision Rel 4.8. Quantitative assessment of microcirculatory parameters was performed offline by frame-to-frame analysis.

Cell isolation and culture techniques

Mouse hepatocytes were isolated based on a two-step collagenase perfusion technique. In brief, mouse livers were perfused through the portal vein first with perfusion buffer solution and then with liver digest medium. Hepatocyte suspensions were separated by centrifugation at 50 g for 4 min and washed three times, and then 1×10^6 cells were plated onto 60 mm collagen-coated tissue culture plates in DMEM with 10% fetal bovine serum, 100 g/ml penicillin, and 100 g/ml streptomycin. After overnight culture, 1mM MLT was given to each well, the whole culture plate was placed into a hypoxia incubator (90% N2, 5% CO_2 and 1% O_2) at 37°C for 4 hours, and then switch to normoxia incubator (5% CO_2 and air) at 37°C for 3 hours. MLT was supplementaed again immediately before moving to normoxia incubator.

Statistical Analysis

Data are expressed as mean ± SD. Differences between groups were evaluated by an unpaired t test, Kaplan-Meier curve were used to analyze survival data in I/R+exPH and SFS-LT groups. Statistical analysis was performed by Prism 6.0 (GraphPad) and differences were considered statistically significant at $P < 0.05$.

Results

Does MLT reduce hepatic injury in IR+PH mice in a dose dependent manner?

Due to experimental simplicity and stability, I/R+PH model was firsty performed to mimic the model of partial liver transplantation (Figure 1A). To provide evidence whether MLT protects liver damage after ischemic reperfusion plus liver resection, we performed a dose-response experiment using MLT at different concentrations of 0.08, 0.4, 2 and 10 (mg/kg). A bolus was applied in I/R+PH mice 15 min before surgery and immediately after reperfusion. , Level of AST and ALT, markers of liver injury in serum were measured at 1, 6 and 24 hours postoperatively. There were no significant differences of liver injury among the 5 groups at 1 and 6 hours. At 24 hours, however, we observed a sharp decrease in both serum AST and ALT with 10 mg/kg MLT treatment (Figure 1B). These results indicated that the highest dose of MLT (10 mg/kg) exhibited significant hepatic protective effects compared with the lower doses; therefore, we used 10 mg/kg MLT in the subsequent experiments.

MLT ameliorates hepatic injury in I/R+PH mice

I/R+PH surgery was performed in mice with vehicle or MLT treatment. Serum and remnant liver tissue samples were harvested at 1, 3, 6, 16, 24 and 48 hours after reperfusion. In the control group, AST and ALT reached the peak at 16 and 24 hours respectively, and stayed at a high level till 48 hours. In contrast, MLT significantly decreased the level of both markers at 16 and 24 hours and returned to basal levels at 48 hours post-I/R+PH (Figure 3A).

HMGB1 is a protein located in the nucleus, which plays a role in transcription. Under the stress of IRI, HMGB1 could translocate to the cytoplasm and further into circulation. In I/R+PH controls, translocation of HMGB1 from the nucleus to the cytoplasm was observed after 24 hours of reperfusion. This translocation can be associated with hepatocyte injury, which coincides with the increased AST and ALT levels at 24 hours. On the contrary, a dramatic inhibition of cytoplasmic translocation was found by MLT treatment (Figure 3B). In circulation, two peaks of HMGB1 were observed. The first peak appearing at one hour, might be attributed to active secretion by innate immune cells, the second peak around 24 hours was most likely from hepatocyte injury, consistent with the elevation of AST and ALT levels at 24 hours. The serum level of HMGB1 was significantly reduced by MLT treatment at 16, 24 and 48 hours (Figure 3C), indicating a hepato-protective role of MLT [21].

DNA double-strand breaks (DSBs) is a lethal process of cell damage. Therefore, we evaluated Gamma-H2A.X, a biomarker of DSBs, to further investigate the protective effect of MLT. After 3 hours post-I/R+PH, we observed

scattered hepatocytes positive for Gamma-H2A.X staining in vehicle treated mice. Livers from MLT treated mice demonstrated scarcely positive hepatocytes (Figure 3D). These findings indicate that MLT protects hepatocytes by reducing IRI injury, inhibiting the cytoplasmic translocation of HMGB1 and preventing DNA damage in I/R+PH model.

Is the protective effect of MLT in I/R+PH mediated by MLT receptors?

To assess the involvement of MLT receptors in hepatic protection, mice were injected with luzindole, a specific antagonist to both Melatonin receptor 1A (MT1) and Melatonin receptor 1B (MT2). If the protective effect of MLT were mediated by MLT receptors, the protective effect would be eliminated by luzindole treatment. However, the level of AST and ALT still significantly dropped with the blockage of MT1 and MT2 when compared with the control groups (Figure 2). This data indicates that the protective role of MLT in I/R+PH mice is independent of MLT receptors.

MLT enhances IL-6, TNF-α release and liver regeneration in I/R+PH model

To investigate the role of MLT in liver regeneration, we further tested the level of IL-6 and TNF-α, two cytokines that are important in initiating liver regeneration after hepatic injury or tissue loss. Previous reports indicated that MLT suppresses IL-6 and TNF-α in hepatic IRI mice [16]. Surprisingly, dramatic elevation of both cytokines were observed in liver tissue at 1, 3 and 6 hours post-I/R+PH, when mice were treated with MLT. Both cytokines returned to the basal level at 24 hours (Figure 4A). A release of serum IL-6 was also increased by MLT treatment at 3 and 6 hours (Figure 4B). We have previously shown that cold ischemic injury inhibits liver regeneration promoting SFSS after transplantation in a IL-6 and TNF-α dependent mechanism [22]. This study confirms further that the early increase of IL-6 and TNF-α by MLT treatment plays an important role in priming hepatocytes to enter the cell cycle for proliferation.

We then performed proliferating cell nuclear antigen (PCNA) and phospho-histone 3 (pH3) immunohistochemistry staining 48 h after surgery. PCNA is a classic marker for activation of cell proliferation while PH3 is a more exclusive marker for cell mitosis. We observed significantly increased proliferating hepatocytes by staining for both markers in the MLT treated group (Figure 4C-D), suggesting that MLT enhances entry into the cell cycle is in I/R+PH. This finding was supported by estimations of liver/body ratio, which showed that MLT treated animals had a significantly improved liver weight compared with the control group (Figure 4E).

MLT improved hepatic microcirculation and survival rate in I/R+exPH model

MLT enhances liver regeneration in I/R+PH mice, which we used as a model of partial liver transplantation. To further explore the potential of MLT to protect from combined damage of ischemia and hepatectomy, we establishe an extended resection with a remnant of 20% liver volume. This I/R+exPH approach was used to test SFSS in mice. (Figure 5A).

A successful liver transplant relies on well preserved microcirculation. We have previously reported that intact microcirculation is essential for hepatic recovery after IRI [20]. SFSS liver grafts display poor sinusoidal perfusion because of portal hypertesion and IRI [23]. We applied intravital microscopy (IVM) in I/R+exPH mice 3 hours after surgery. In the control group, we observed obstructed sinusoidal networks, which displayed reduced sinusoidal perfusion and increased adherent leukocytes in hepatic microcirculation 3 hours after surgery. On the contrary, MLT treatment retained almost intact sinusoid perfusion and attenuated leukocytes adhesion (Figure 5B-C).

In order to test whether treatment with MLT had a beneficial outcome, a survival experiment of I/R+exPH was performed. In the control group, all animals died in 2 days after surgery. With a perioperative treatment of MLT, the survival rate had significantly increased from 0% to 50% 7 days postoperatively (Figure 5D). It demonstrated that MLT contributes to preserved hepatic microcirculation and improved animal survival.

MLT increases recipients' survival in SFS-LT mice

I/R+PH and I/R+exPH models partially simulate partial liver transplantation, but they do not completely represent transplantation of the liver because of the absence of cold ischemia and a complicated surgery resulting in more injury. We therefore tested the effect of MLT in SFS-LT model, transplanting 30% graft (Figure 6A). This model of SFS liver transplantation reflects more closely the clinical situation of SFSS [24]. 30% liver (median lobe) grafts were transplanted in an orthotopic location in recipient mice. Recipient mice were divided into MLT or vehicle (control) treated groups. In the control group, all animals died within 4 days due to liver failure caused by SFSS. MLT treatment ameliorated liver failure and raised the survival rate of recipients from 0% to 57% after 7 days of transplantation (Figure 6B). The result indicates that MLT significantly improves survival of recipient mice after SFS-LT.

MLT activates IL-6, TNF-α, HGF and gp130/STAT3 pathway after SFS-LT

To explore the mechanism why MLT improves recipients' survival in SFS-LT, we investigated the expression of cytokines and growth factors that might regulate liver regeneration at early time points after SFS-LT. Liver

tissues were obtained at 3 and 6 hours after transplantation, the mRNA expression of IL-6, TNF-α and HGF were measured by RT-qPCR. In the MLT treated group, a 2-fold higher induction of IL-6 was observed after 3 hours, and after 6 hours a 3-fold increase of IL-6 levels was demonstrated in MLT treated group compared to control mice (Figure 7A). The mRNA expression of TNF-α and HGF also significantly increased at 6 hours after surgery in MLT treated group (Figure 7B). The elevation of IL-6 level in SFS-LT model was consistent with our findings in the I/R+PH model, which indicates an important role of MLT in initiating liver graft regeneration. Thus, we expected that IL-6 signaling is crucial for the liver protective effect of MLT in SFS-LT.

Therefore, we further tested the expression of IL-6 pathway in liver grafts on the protein level. IL-6 binds to Interleukin-6 receptor (IL-6R), the signaling is subsequently initiated upon association of the IL-6/IL-6R complex with a transmembrane receptor protein, glycoprotein (gp) 130. This leads to the activation of signal transducer and activator of transcription (STAT) 3. STAT3 is an essential factor for liver regeneration and its activity is controlled by phosphorylation. In line with the increase of IL-6 mRNA expression, we observed stronger activation of gp130 and phosphorylation of STAT3 in MLT treated group vs. controls after 3 hours of SFS-LT (Figure 7C). We conclude that IL-6/gp130 dependent STAT3 pathway was activated by MLT.

Blockage of IL-6 signaling suppresses hepatocyte proliferation and eliminates hepatic protective effect of MLT

To determine whether interruption of IL-6 signaling in I/R+PH mice might eliminate the positive effect of MLT in liver regeneration, IL-6-/- mice were applied in both MLT treated and control groups. When control mice were subjected to I/R+PH, pH3 positive hepatocytes were slightly less than in wild-type mice. Furthermore, MLT treatment alone couldn't improve the regenerative capability of IL-6-/- mice post-I/R+PH. In order to restore IL-6 level, recombinant mouse IL-6 protein (rIL-6) was injected 30 minutes before surgery, we observed significantly increased pH3positive hepatocytesin IL-6-/- mice treated with both MLT and rIL-6 (Figure 8A). These data suggested the protective effect of MLT required activation of IL-6 signaling in liver regeneration.

It has been demonstrated that gp130 is an indispensable element of IL-6 signaling [25]. To further double-check the importance of the IL-6/gp130-Stat3 pathway in our study, gp130 was inhibited using a specific inhibitor, SC144. The regenerative capacity in I/R+PH model and mouse survival rate in I/R+exPH and SFS-LT models were assessed. The capacity of liver regeneration in mice that received SC144 injection was impaired even with the treatment of MLT. This was demonstrated by both PNCA and pH3 staining 48 hours post-I/R+PH (Figure 8B). In I/R+exPH and SFS-LT models, none of the mice who were treated with SC144 together with MLT could survive more than 3 days. Thus, the hepatic protective effect of MLT has been completely eliminated in both

models (Figure 9A-B). Overall, our findings indicate that IL-6/gp130 dependent STAT3 pathway is crucial for the protective effect of MLT.

Discussion

The SFSS is mainly caused by defective regeneration and ischemic injury after transplantation of a small liver graft in the setting of living-donor liver transplantation [26]. This study demonstrates that MLT promotes graft regeneration and reduces IRI injury in SFS liver transplant by activating through IL-6/gp130-Stat3 pathways, thereby improving significantly recipient's survival of SFS liver transplant. It may open a new avenue to rescue SFSS in LDLT.

MLT has shown a strong antioxidative property by protecting IRI in hepatic IR models [14-16]. Our results indicated a striking protective effect of MLT in SFS liver graft injury by decreasing the levels of AST and ALT, inhibiting HMGB1 translocation and preventing DNA double-strand breaks. Besides, the results of primary mouse hepatocyte culture experiment also supported a direct hepatocyte protective role of MLT (supplement data). Notably, this protective effect of MLT acts in a dose dependent manner, independent of MLT receptors (Fig. 1 and 2). These data are in accordance with previous findings, which can be partially explained by the anti-oxidative effect of MLT [27]. Previous reports showed that the hepatic protective effect of MLT in IRI models were associated with a reduction of IL-6 and TNF-α, we found that MLT significantly elevated the levels of IL-6 and TNF-α at 1-6 hours in the liver and serum after I/R+PH and SFS-LT surgery. This is in accordance with the theory that TNF-α dependent IL-6 secretion in the early phase is required for liver regeneration and protection [28].

First of all, IL-6 and TNF-α are key cytokines in initiating liver regeneration by priming hepatocytes to enter the cell cycle [2]. Second, pro-inflammatory IL-6 also coordinates anti-inflammatory activities which are essential for the resolution of inflammation. Therefore, IL-6 has context-dependent pro- and anti-inflammatory properties. Once the IL-6 receptor complex is activated, there are multiple downstream events that allow IL-6 to mediate its diverse effects [29]. Oxidative and Fas-mediated damage play prominent roles in hepatic injury, resulting in both hepatic necrosis and apoptosis. A functioning IL-6 receptor depends on the formation of an IL-6–IL-6R–gp130 complex. Subsequently, Janus kinase-1 (JAK-1) induces the release of STAT3 from the GP130 complex. After dimerization of STAT3, the dimer is able to transfer to the nucleus for activation of a select program of genes. [30]. Among the many functions, IL-6 signaling provides hepatoprotection against Fas-mediated apoptotic liver

damage by inactivation of caspases and reduction of reactive oxygen species [31]. IL-6 is furthermore involved in controlling hepatocyte proliferation and cell protection after partial hepatectomy (PH) [32]. The signaling via gp130 is also essential for hematopoiesis, cell survival and growth. Indeed, our study revealed that MLT elevated the levels of IL-6 and TNF-α, activated gp130-Stat3 signaling in models of I/R+PH and SFS-LT, and eventually resulted in promoting liver regeneration, ameliorating hepatic ischemia injury, and increasing animal survival significantly. Although the protective effect of MLT against IRI in I/R+PH mice occurred 10 hours later than the previous reports for a hepatic IRI, the reduction of AST, ALT and HMGB1 in our study were impressively significant (Fig. 3 A, B and C). More importantly, the dramatically elevated IL-6 and TNF-α level in the early time points play an important role in protecting hepatocytes and initiating liver regeneration, since liver regeneration is the most essential reaction to liver injury [33, 34]. Another study using PH/LPS challenge revealed that the activation of gp130-dependent STAT3 signaling is involved in DNA synthesis and protects hepatocyte proliferation during stress conditions. HGF and IL-6, promote hepatocyte survival by stimulating liver regeneration and providing hepatoprotection [35]. These findings are consistent with our observations.

It has been demonstrated that IL-6 pathway is critical for cell proliferation and animal survival [36, 37]. With SFS-LT model, we examined the activation of IL-6 downstream pathway at the protein level by the treatment of MLT. We observed an upregulation of gp130 expression and a rising STAT3 phosphorylation after 3 hours of transplantation. The finding with SFS-LT model is in agreement with the results of our I/R+PH and I/R+exPH models: the elevated level of IL-6 resulted in reduced hepatic injury, enhanced liver regeneration and improved survival. They are also in accordance with the previous reports that IL-6 pathway is crucial for the initiation of liver regeneration [38] and hepatocyte protection [39].

To verify the role of IL-6, we silenced IL-6 by applying IL-6-/- mice in I/R+PH experiment. Interestingly, the regeneration promoting effect of MLT was abolished. Both pH3 and PCNA immunostaining displayed unchanged activation of hepatocyte proliferation. inIL-6-/- mice in the presence or absence of MLT treatment. The regeneration promoting effect of MLT could only be restored by concurrent administration of MLT and recombinant IL-6 (Fig. 8A), suggesting that IL-6 (including IL-6-Il-6R-gp130) is required for MLT mediated hepatoprotective effects and regeneration after I/R+PH surgery.

The gp130 dependent Stat3 signaling is a decisive factor for liver regeneration and protection [40]. To confirm the importance of the gp130-Stat3 pathway, we interrupted gp130 by using SC144 in I/R+PH, I/R+exPH and SFS-LT experiments. SC144 specifically blocks gp130 protein without influencing IL-6R [41]. Blocking gp130 eliminated the effect of MLT in promoting liver regeneration. MLT plus SC144 treated I/R+PH animals showed

impaired hepatocytes proliferation demonstrated by PCNA and pH3 staining (Fig. 8B). Of note, all animals treated with MLT plus SC144 died within 3 days after I/R+exPH and SFS-LT surgery (Fig. 8C, D), more than half of them are expected to survive over 7 days if they were treated with MLT alone. These data demonstrated convincingly that gp130 is essential to rescue SFSS.

It is interesting that MLT upregulates IL-6 and promotes liver regeneration only in the models combined with IRI and PH, but has no effect in pure 70% PH model [42]. Probably 70% PH is a robust liver regeneration model without IRI that does not require protective support. The hepato-protective effect of MLT might be only triggered by serious damage e.g. during a combined insult such as ischemia and resection (IRI plus PH).

Sinusoidal endothelium of SFS liver is subject to an assault of transient portal hypertension and increased portal flow. Thereby a preserved hepatic circulation is vital for a successful liver transplant and liver regeneration [43, 44]. Nitric oxide (NO) cascade and endothelial nitric oxide synthase (eNOS) are well known for their function in endothelium-mediated relaxation of vascular smooth muscle. MLT could reduce vascular resistance, generate NO through constitutive eNOS activation in cold preserved fatty liver [17], augment HO-1 through attenuating inducible nitric oxide synthase (iNOS) protein [16]. These features of MLT suggest that the hepatic function may be improved by MLT via sinusoidal vasodilation [45]. Indeed, hepatic protection by MLT was associated with reduced endothelin and increased NO bioavailability in hepatic sinusoids [46], and MLT augmented the increase of eNOS mRNA level, while it reduced the increase in the iNOS mRNA level during hepatic I/R [16, 47]. Our IVM test revealed that hepatic microcirculation of SFS liver was damaged by the SFSS, resulting in the blockage of more than 40% hepatic sinusoidal circulation. This was accompanied by increased adhesion of leukocytes in postsinusoidal venules. In contrary, MLT treated livers retained almost intact sinusoidal circulation at 3 hours after surgery (Fig. 5 B). Although the methods for continuously monitoring portal pressure and flow are restricted by the size of mice and complexity of surgery, we could demonstrate that MLT improves hepatic microcirculation which is essential to rescue SFSS.

The application of MLT in LDLT seems a more efficient strategy compared with other strategies reported in the literature. First, it is a more effective treatment since MLT exerts multifunctional effects in ameliorating SFSS. We demonstrated that MLT reduces cold and warm IRI, improves hepatic microcirculation and enhances hepatocyte proliferation by activating the IL-6/gp130-Stat3 pathway. Secondly, MLT can be administered in high doses without any sign of side effects. Since it is impossible to induce a significant mortality in mice even with the highest possible dose (800 mg/kg), the IC50 could not be determined [48], demonstrating that MLT can be safely applied with high doses. A clinical study administrated 50 mg/kg MLT to patients who underwent

massive hepatectomy and all the patients tolerated MLT well [49]. Besides, MLT is already used clinically [50]. Thus, transfer to clinical studies and finally as an effective drug in LDLT should not be hampered regulatory obstacles. Therefore, MLT will be an effective, simple, safe and prompt strategy to prevent SFSS in LDLT. The clinical application of MLT may prevent SFSS and reduce the required liver graft volume for successful LDLT. This will have the benefit of expanding the liver organ pool of live donors without compromising their safety. Thus, MLT would eventually reduce the mortality of end-stage liver diseases.

In summary, this study convincingly demonstrated that MLT reverses SFSS by improving IRI injury, augmenting SFS liver graft regeneration via activating IL-6/gp130-Stat3 signaling and preserving intact hepatic microcirculation after SFS liver transplantation in mice. It has clinic translational potential to prevent SFSS in LDLT and save more patients of end stage liver diseases.

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Figure legends

FIG. 1. MLT reduces hepatic injury in I/R+PH mice in a dose dependent manner. (A) I/R+PH mouse model: 30% of the liver (right lobe and caudate lobe) were subjected to 60 min ischemia, then 70% of the non-ischemic lobes (median lobe and left lobe) were resected after 60 min. (B) Response of liver enzyme to different doses of MLT applied to I/R+PH mice for dose-effective experiment. 1h, 6h and 24h were selected as time points to investigate which dose of MLT is protective (n=5 to 6). Error bars represent the standard error, $*p < 0.05$.

FIG. 2. The protective effect of MLT in I/R+PH model is independent of MLT receptors. MLT receptor antagonist, luzindole, was given to animals 30 min prior to I/R+PH, AST and ALT level were used to evaluate the injury of liver. (n=5). Error bars represent the standard error, $*p < 0.05$, $**p<0.01$.

FIG. 3. MLT ameliorates hepatic injury in IR+PH mice. (A) Level of AST and ALT between vehicle and MLT treated groups at different time points after I/R+PH surgery (n=6). (B) Immunohistochemistry of HMGB1 (20×) in vehicle (n=5) and MLT treated (n=6) mice in order to observe its translocation after 24 hours of surgery. (C) ELISA analysis of circulating HMGB1 in vehicle and MLT treated mice at different time points (n=6). (D) Immunohistochemistry staining of Gamma-H2A.X in vehicle (n=5) and MLT treated (n=5) mice to detect DNA double strands breaks after 3 hours of surgery. The corresponding quantifications were shown on the right. Error bars represent the standard error, $*p < 0.05$, $**p<0.01$.

FIG. 4. MLT increases IL-6 and TNF-α release and enhances liver regeneration in I/R+PH model. (A) mRNA Expression of TNF-α and IL-6 in liver tissue in vehicle and MLT treated group (n=6). (B) ELISA analysis of IL-6 in the circulation in vehicle and MLT treated group (n=6). Quantification of (C) PCNA and (D) pH3 positive cells in vehicle and MLT treated group 48 hours post-I/R+PH (n=6), the representative stainings are shown below. (E) The liver weight gain is presented as liver remnant weight to body weight ratio 48 hours post-I/R+PH (n=6). Error bars represent the standard error, $*p < 0.05$, $**p<0.01$.

FIG. 5. MLT improves hepatic microcirculation and mouse survival after I/R+exPH. (A) I/R+exPH mice model: right lobe of the liver was subjected to 60 min ischemia, then the rest of the non-ischemia lobes (median lobe,

left lobe and caudate lobe) were resected after 60 min. (B) Perfused sinusoids (left) and adherent leukocytes (right) 3 hours after I/R+exPH. Arrows indicate adherent leukocytes. (C) Quantified results of perfused sinusoids and adherent leukocytes 3 hours after I/R+exPH (n=3 per group). Data are shown as mean ± standard error. *p<0.05, **p<0.01. (D) Survival rate (7days) after I/R+exPH between vehicle (n=7) and MLT treated (n=8) group, the survival rate is shown as a Kaplan-Meier plot (p=0.0033).

FIG. 6. MLT increases the recipient's survival in SFS-LT mice. (A) SFS-LT mouse model: median lobe of the liver, which accounts for 30% of the total liver volume was harvested from the donor and implanted into the recipient. (B) Survival rate after SFS-LT between vehicle (n=5) and MLT treated (n=7), shown as a Kaplan-Meier plot (p =0.0027).

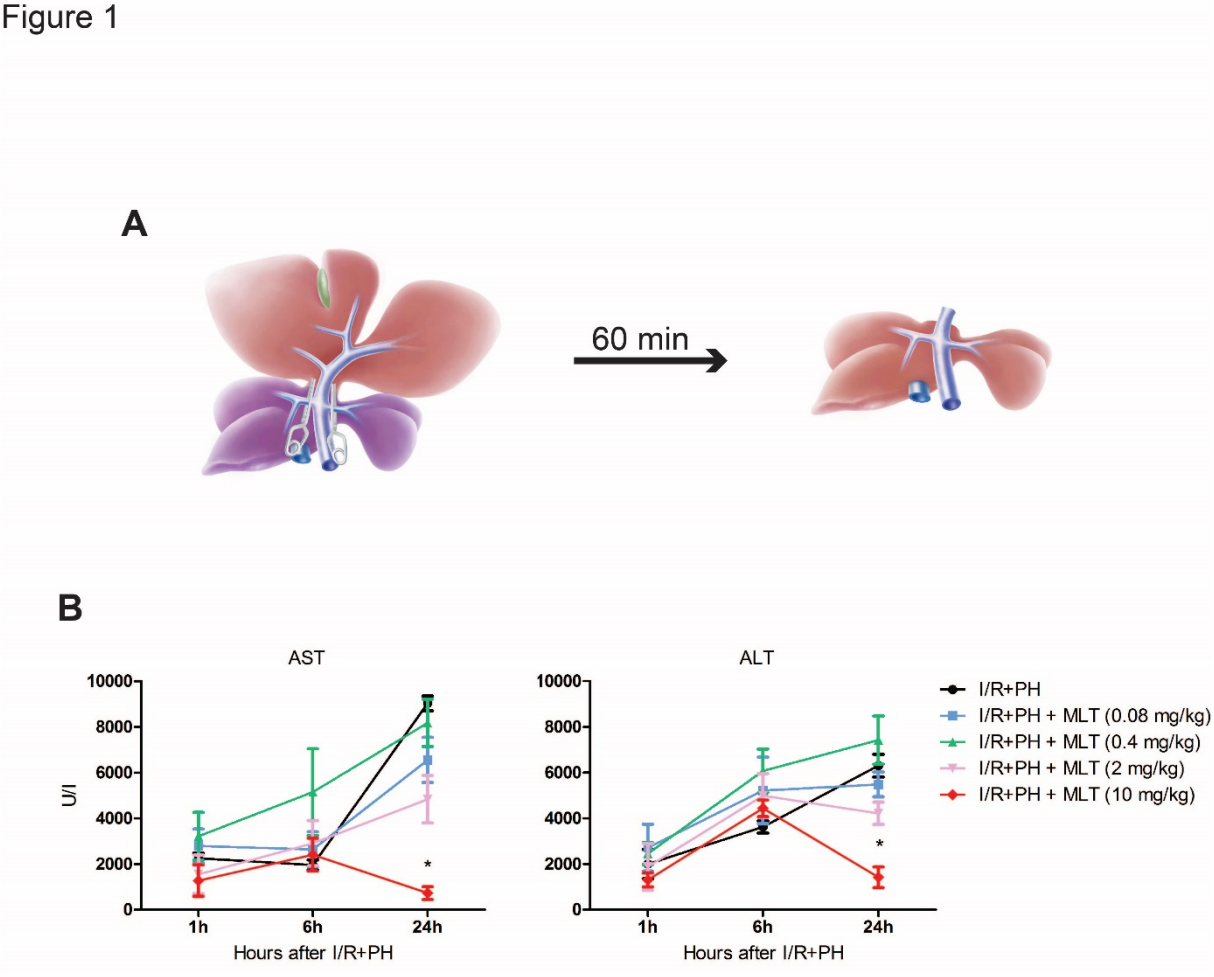
FIG. 7. MLT activates IL-6 / gp130-STAT3 pathway after SFS-LT. (A) Hepatic mRNA expression of IL-6, TNF-α, and HGF in vehicle and MLT treated mouse livers (n=5) 3 and 6 hours post-SFS-LT. (B) Western blot analysis of gp130, p-STAT3 (Tyr-705), STAT3 and b-tubulin at 3 hours post-SFS-LT in livers of vehicle and MLT treated mice (n=5). Values are normalized to b-tubulin. Data are shown as mean ± standard error. *p < 0.05.

FIG. 8. Blockage of IL-6/gp130-Stat3 signaling suppresses hepatocyte proliferation and eliminates the hepato-protective effect of MLT. (A) Immunostaining of pH3 in livers of IL-$6^{-/-}$ mice 48 hours post-I/R+PH Mice were treated with MLT or rIL-6 alone or a combination of MLT and rIL-6 (n=6 per group) respectively. (B) Immunostaining and quantification of PCNA and pH3 positive cells in mice 48 hours post-I/R+PH, mice were treated with vehicle or MLT or MLT + SC144 (n=6 per group). Error bars represent the standard error. *p < 0.05. (C) Survival rate after I/R+exPH mice among vehicle (n=7), MLT (n=8) and MLT+SC144 treated (n=6) groups, shown as a Kaplan-Meier plot (p=0.009). (D) recipient survival rate in SFS-LT mice among vehicle (n=8), MLT (n=7) and MLT+SC144 treated (n=5) groups, shown as a Kaplan-Meier plot (p=0.0095).

Supplement FIG. MLT reduces hepatic enzyme AST, ALT release in primary hepatocyte culture. The level of liver enzyme in cell culture medium when hepatocytes were subjected to 4 hours hypoxia plus 3 hours

reoxygenation. Hepatocytes were treated with either vehicle (n=5) or 1 mM MLT (n=5). Data are shown as mean ± standard error. *$p < 0.05$

Figure 1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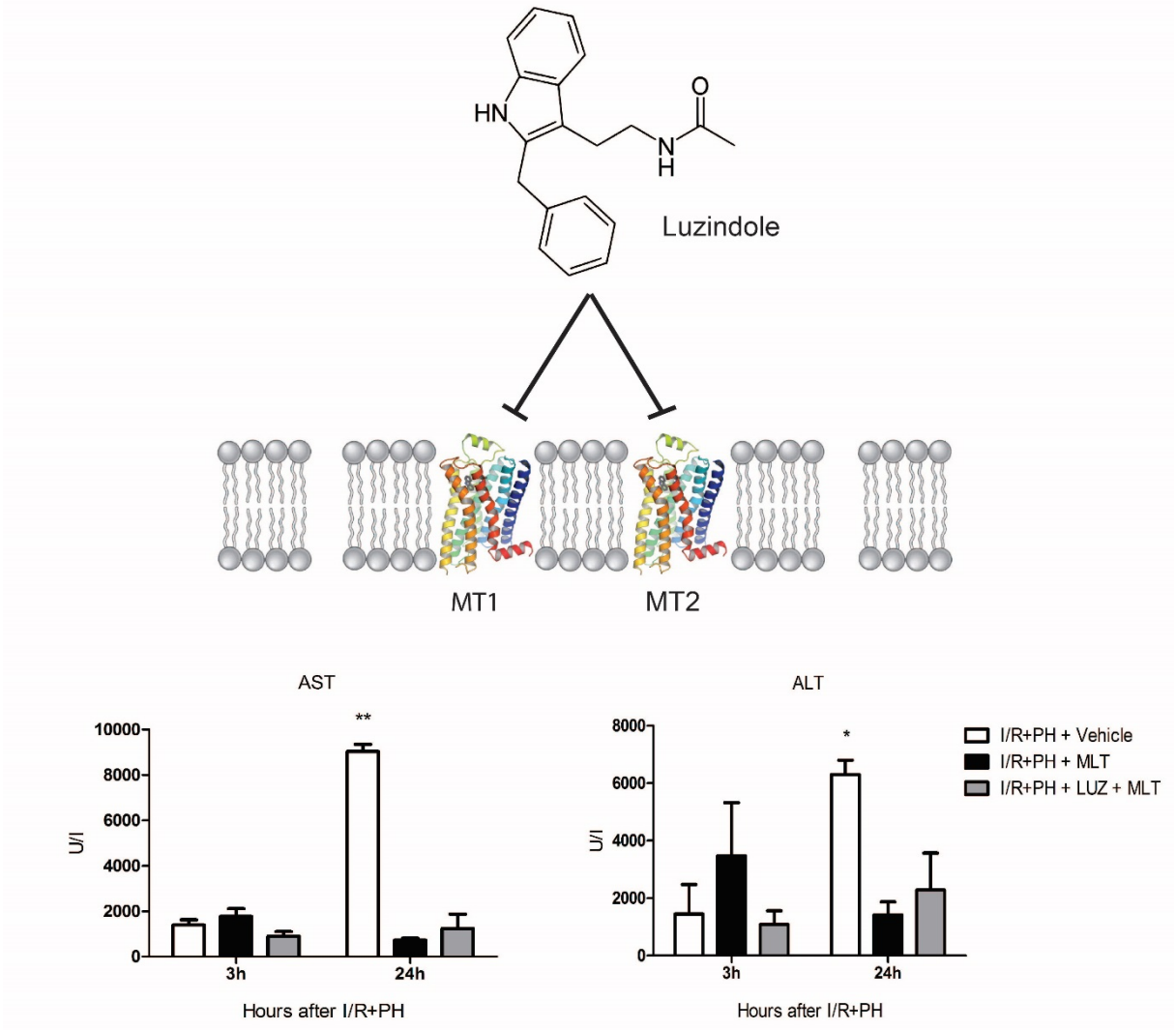
I/R+PH
I/R+PH + MLT (0.08 mg/kg)
I/R+PH + MLT (0.4 mg/kg)
I/R+PH + MLT (2 mg/kg)
I/R+PH + MLT (10 mg/kg)

Figure 2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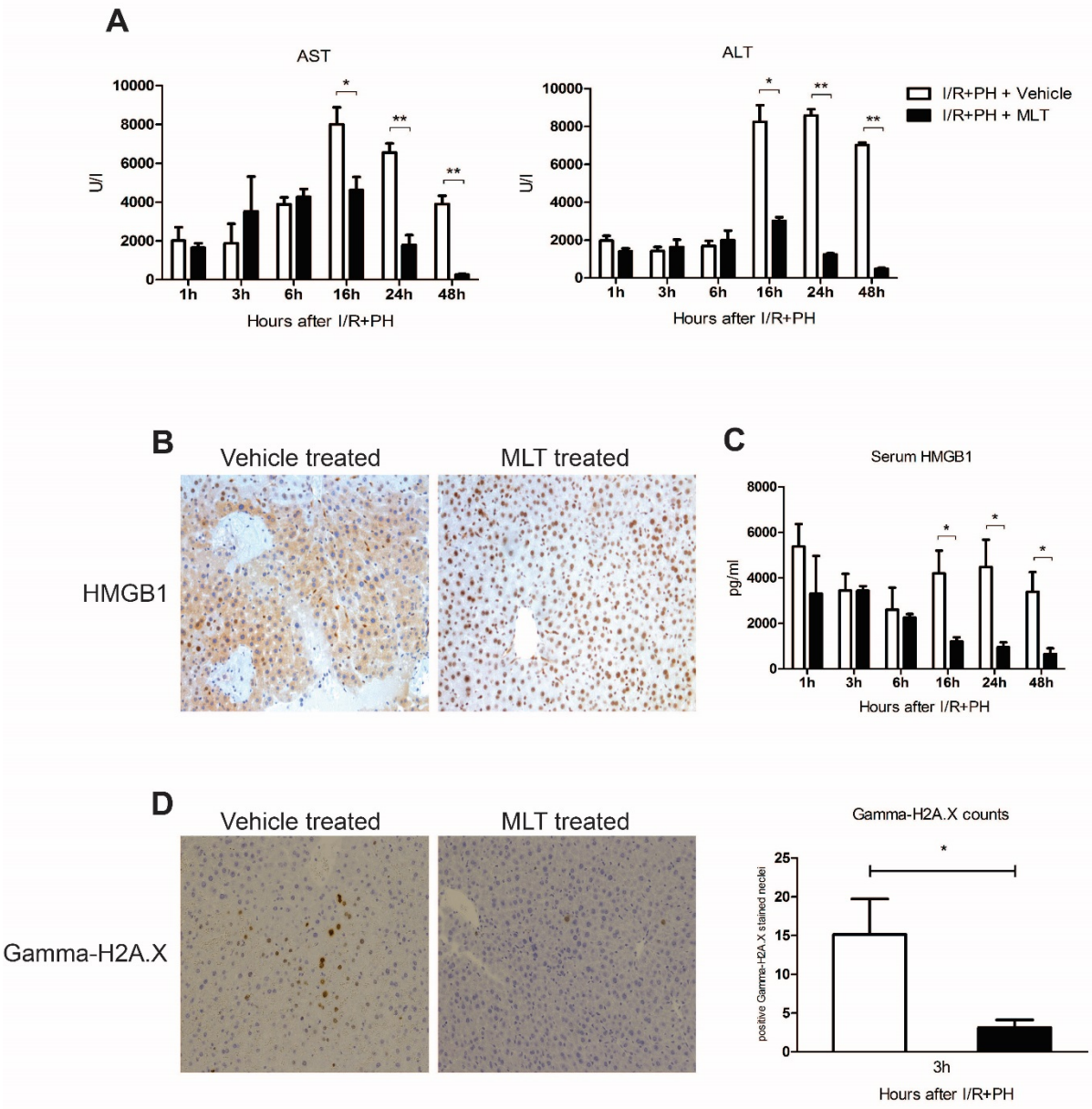
I/R+PH + Vehicle
I/R+PH + MLT
I/R+PH + LUZ + MLT

Figure 3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I/R+PH + Vehicle
I/R+PH + MLT
B
Vehicle treated
MLT treated
HMGB1
C
Serum HMGB1
pg/ml
D
Gamma-H2A.X
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Figure 4

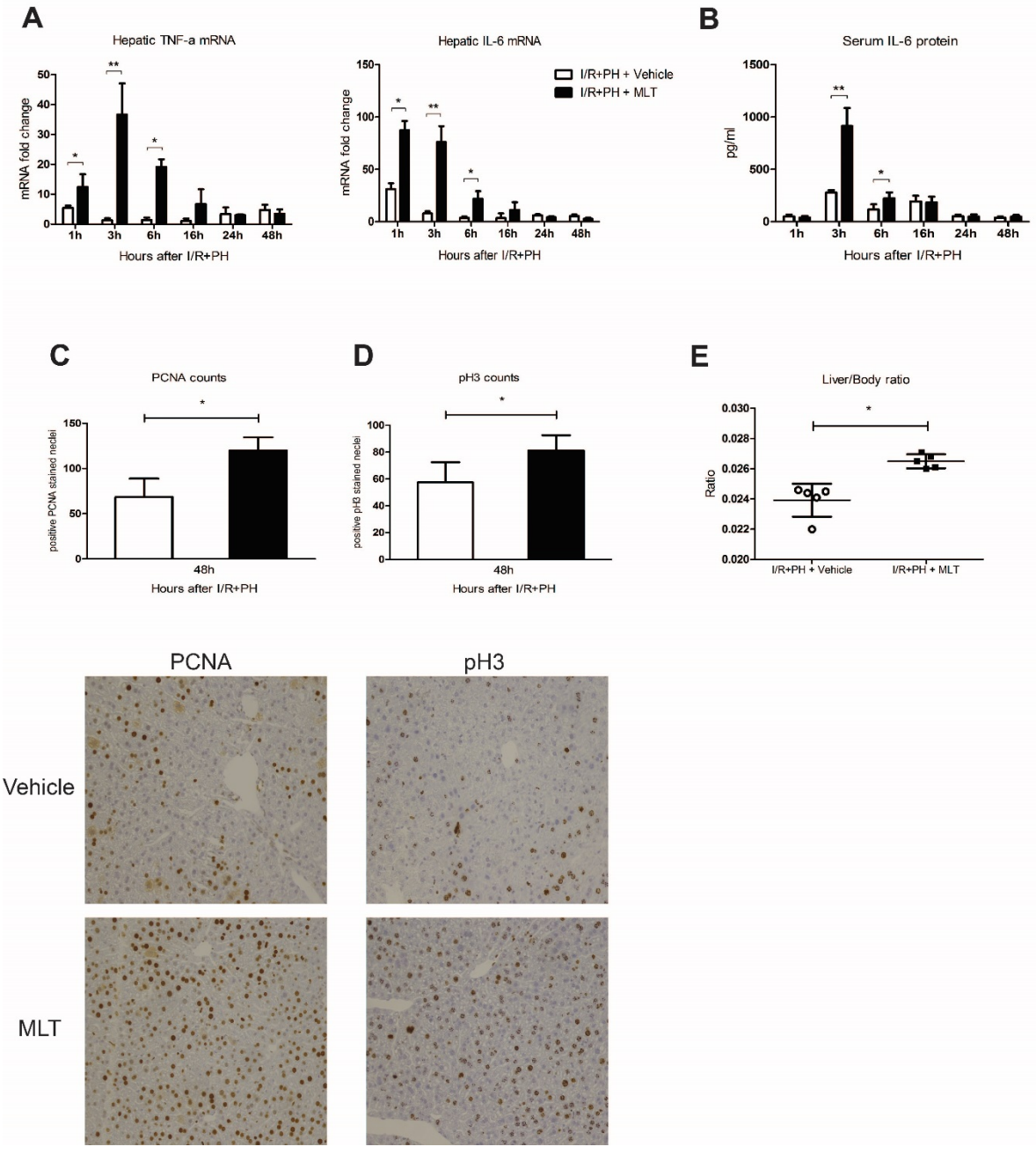
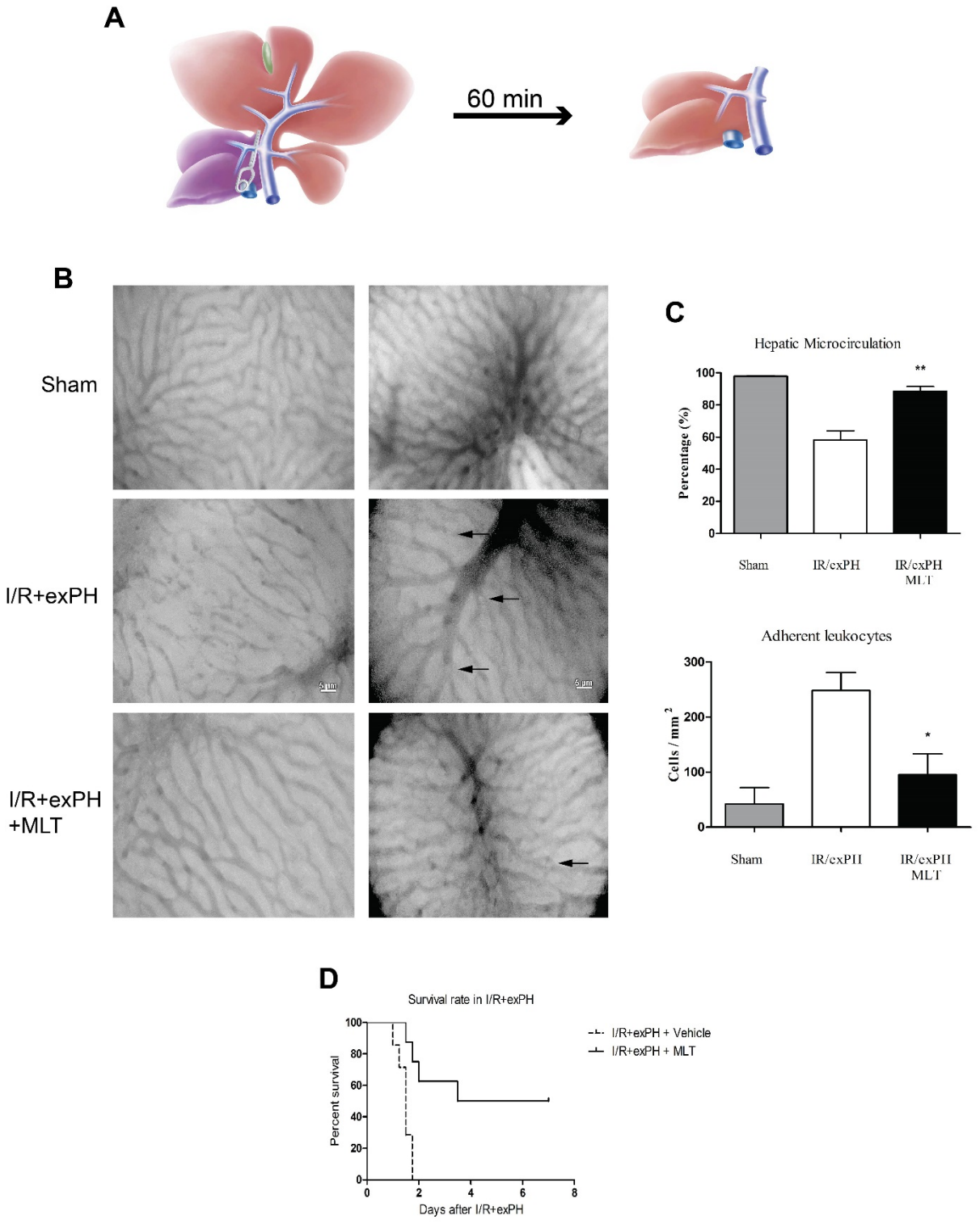

A
Hepatic TNF-a mRNA
mRNA fold change
Hours after I/R+PH
Hepatic IL-6 mRNA
I/R+PH + Vehicle
I/R+PH + MLT
B
Serum IL-6 protein
pg/ml
C
PCNA counts
positive PCNA stained nedei
D
pH3 counts
positive pH3 stained nedei
E
Liver/Body ratio
Ratio
PCNA
pH3
Vehicle
MLT

Figure 5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I/R+exPH
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+MLT
C
Hepatic Microcirculation
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Sham
IR/exPH
IR/exPH
MLT
Adherent leukocytes
Cells / mm 2
300
200
100
0
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Sham
IR/exPH
IR/exPH
MLT
D
Survival rate in I/R+exPH
Percent survival
100
80
60
40
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2
4
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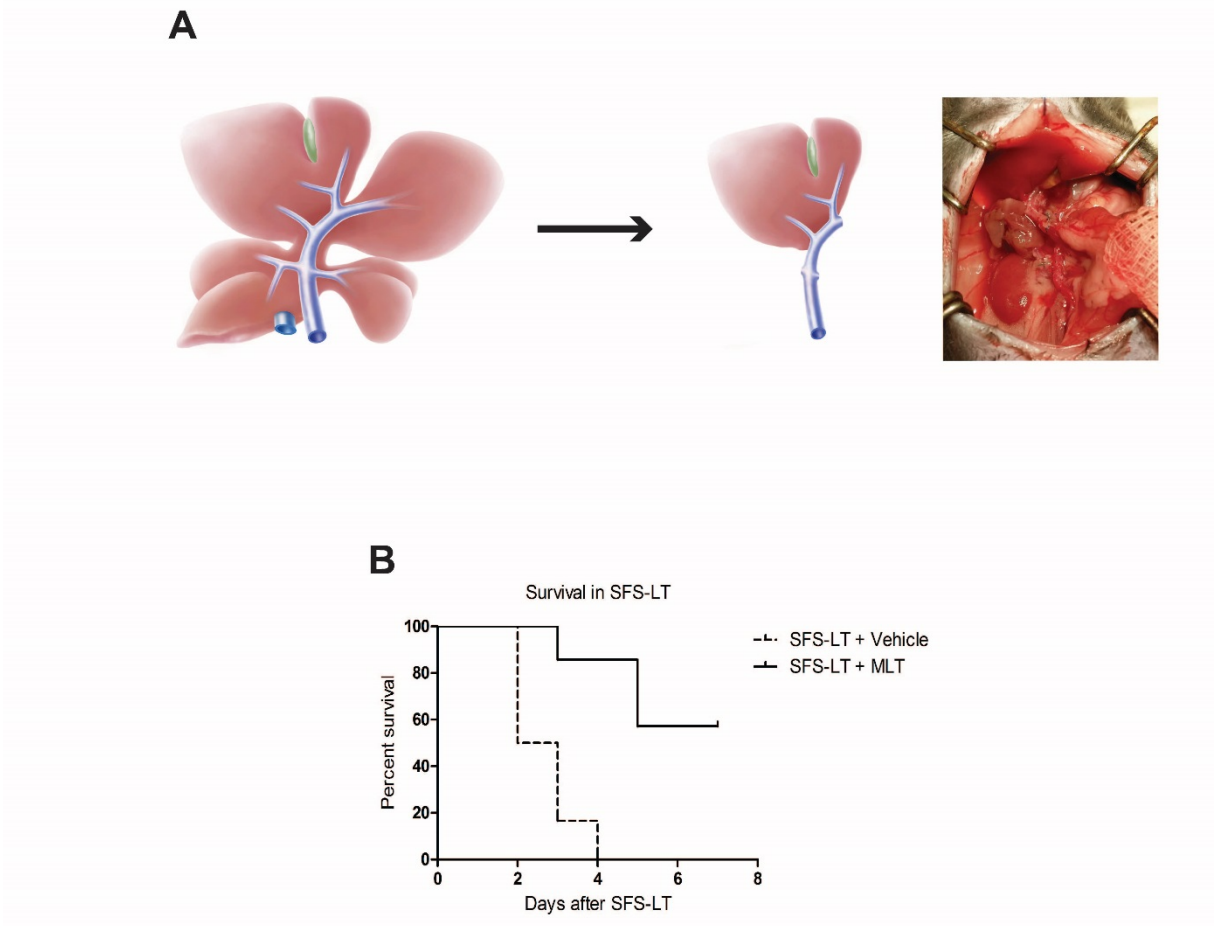
8
Days after I/R+exPH
I/R+exPH + Vehicle
I/R+exPH + MLT

Figure 6


A
B
Survival in SFS-LT
SFS-LT + Vehicle
SFS-LT + MLT
Percent survival
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2
4
6
8
Days after SFS-LT

Figure 7

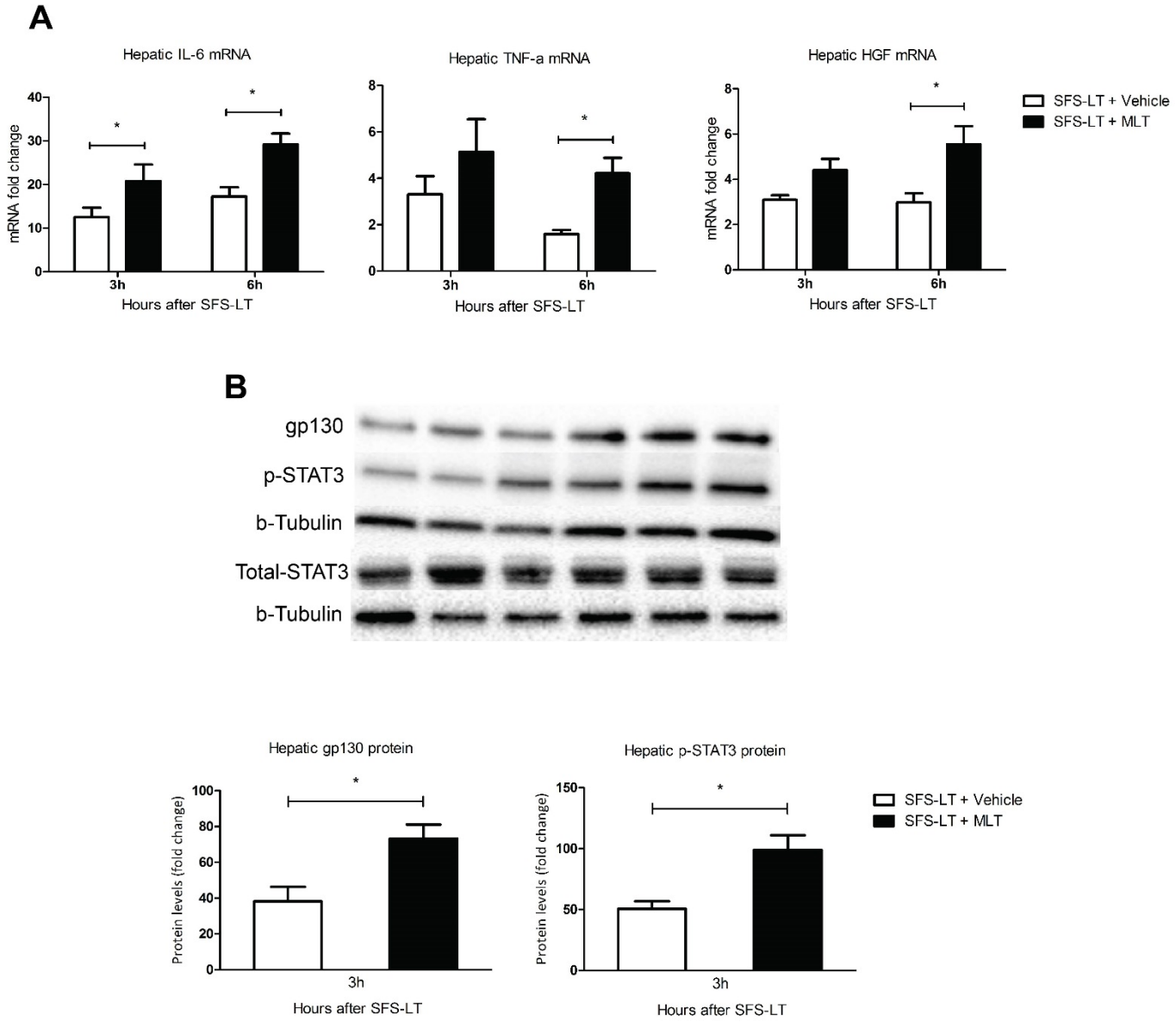
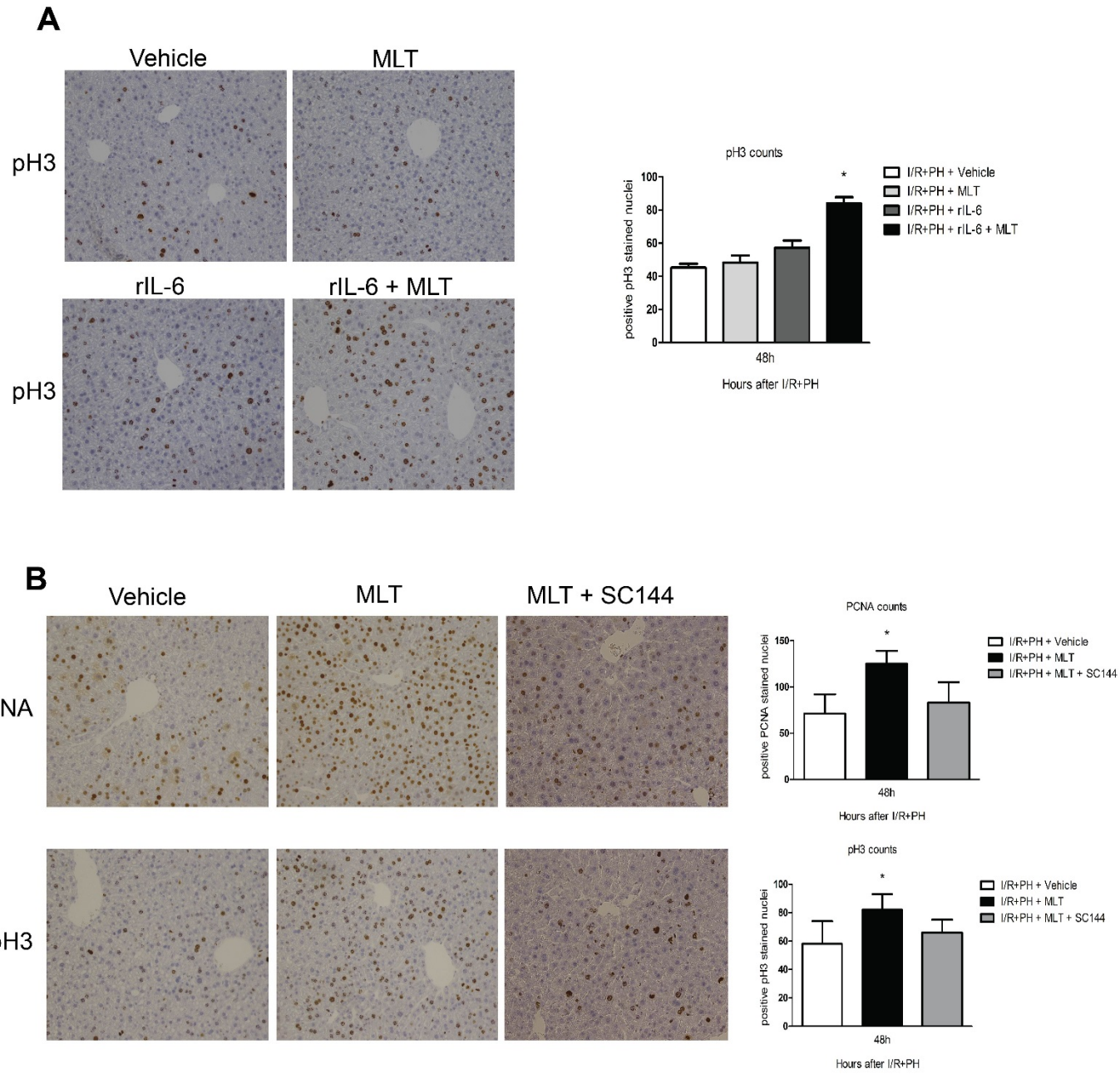

A
Hepatic IL-6 mRNA
mRNA fold change
Hours after SFS-LT
Hepatic TNF-a mRNA
Hours after SFS-LT
Hepatic HGF mRNA
mRNA fold change
Hours after SFS-LT
SFS-LT + Vehicle
SFS-LT + MLT
B
gp130
p-STAT3
b-Tubulin
Total-STAT3
b-Tubulin
Hepatic gp130 protein
Protein levels (fold change)
Hours after SFS-LT
Hepatic p-STAT3 protein
Protein levels (fold change)
Hours after SFS-LT
SFS-LT + Vehicle
SFS-LT + MLT

Figure 8


A
Vehicle
MLT
pH3
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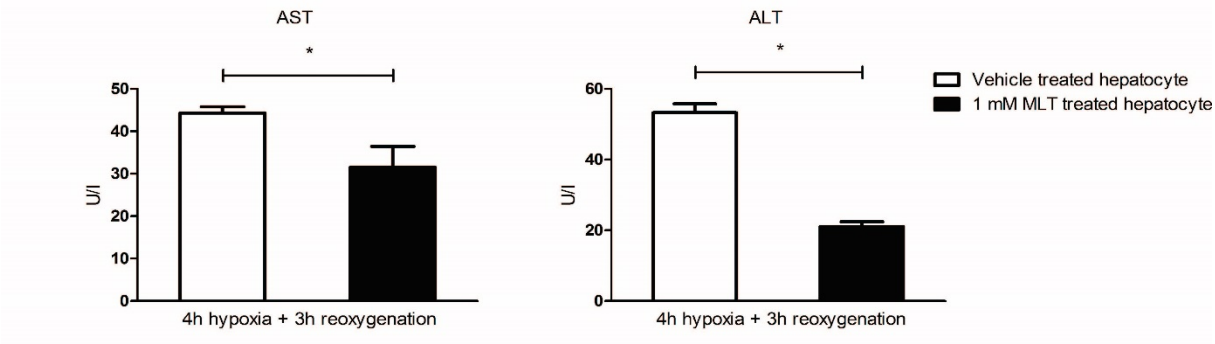
SFS-LT + MLT + SC144
Days after SFS-LT

Supplement figure 1



6. Manuscript B

Title:

Time of Operation and Feeding Behavior Influence the Short-term Outcome after Hepatic Ischemia Reperfusion Injury

Short title:

Circadian rhythm influences hepatic ischemia reperfusion injury

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Abbreviations:

Abbreviations used in this paper: *ALT, alanine aminotransferase; AST, Aspartate aminotransferase; Bmal1 (Arntl), Aryl hydrocarbon receptor nuclear translocator-like protein 1, Cry1, cryptochrome protein 1; Cry2, cryptochrome protein 1, Per1, period circadian protein homolog 1, Per2, period circadian protein homolog 2, HMGB1, High-Mobility-Group-Protein B1; IL-6, interleukin-6; IL-6R, Interleukin-6 receptor; MLT, melatonin; TNF-α, tumor necrosis factor alpha; ZT, Zeitgeber*

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Disclosures:

The authors have nothing to disclose

Contribution:

This is the side project during my research work, I have performed all the animal surgeries and most biological analysis. I solely designed the whole project and drafted this manuscript.

Time of Operation and Feeding Behavior Influence the Short-term Outcome after Hepatic Ischemia Reperfusion Injury

Zhuolun Song, Rolf Graf, Eleonora Maurizio, Bostjan Humar, Pierre-Alain Clavien, Yinghua Tian

Abstract

Background: Liver transplantation surgeries require speedy implantation to guarantee the vitality of the donor livers. Due to the unpredictable availability of donor organs, the operation could take place at any time of the day. The aim of this study it to investigate whether the timing of surgery and short-term fasting play roles in hepatic ischemic reperfusion injury (IRI).

Methods: Mice were subjected to 70% hepatic ischemia for 60 min and followed by 6h reperfusion at different time points of the day. Serum and histological markers of hepatic injury were measured. The influence of surgery on the expression of circadian genes was assessed. Standardized time 7:00 (ZT7) and 18:00 (ZT18) were chosen for further analysis. A physiological s dose of melatonin (MLT) was given to mice before surgery to mimic the endogenous peak of melatonin. The role of short-term fasting on hepatic injury was evaluated by applying 6h fasting to the mice prior to surgery at ZT7 and ZT18.

Results: Our data show that hepatic IRI differs when operating at different time of the day. Nighttime operations show less injury than daytime operations. A significant protective effect was observed when operating at ZT18 compare with ZT7. A shift of the expression of major circadian genes was observed by hepatic IRI surgery. Those observations were not affected by supplementing a physiological dose of MLT. We also found that 6h fasting before surgery exhibits completely different impact on hepatic injury between ZT7 and ZT18. Hepatic IRI at ZT7 was attenuated while hepatic IRI at ZT18 was worsened.

Conclusions: Operation time and short-term fasting play important roles in hepatic IRI. This impact is probably dependent on circadian genes but not on endogenous MLT levels.

Key words: Hepatic ischemia reperfusion injury; Circadian rhythm; Melatonin; Fasting.

Introduction

Hepatic ischemia reperfusion injury (IRI) is one of the main causes of postoperative hepatic dysfunction and other complications after major hepatic resection. This injury results from transient interruptions of the blood flow to the liver to prevent blood loss. In the case of liver transplantation, hepatic IRI, which is inevitable, is a major contributor of poor outcome in the recipients. In order to shorten the cold preservation time and decrease the chance of developing primary graft non-function, liver transplantation surgery requires speedy organ retrieval, transportation and implantation. Due to the unpredictable availability of cadaveric donor livers, surgery of liver transplantation has to be performed at any time of the day. One study showed that nighttime liver transplantation was associated with greater risk of early death than those who underwent liver transplantation during daytime [1]. Although surgical or anesthetic skills at night may influence the outcome, some other factors such as circadian rhythm might also be involved in this process.

Circadian rhythm is a biological process which is generated from a transcription-translational feedback loop. A number of transcription factors, also known as circadian genes, are essential for the formation of circadian rhythm by expression in a cyclic fashion. The organism is synchronized to a 24-hour light/dark rhythm cycle by the circadian system [2,3]. The circadian rhythm regulates many physiological functions in living systems, including sleep-wake cycle, feeding habit, body temperature, blood pressure, hormone regulation, drug metabolism, cell proliferation and so on [4]. The circadian gene expression can be disrupted by genetic or environmental factors This type of disruption will result in diverse physiological disorders and eventually lead to diseases [5]. The liver is an important organ, which is responsible for various body functions and it bears the highest number of active circadian genes and have a crucial role in liver homeostasis [6]. In recent years, research has identified several molecular mechanisms which link circadian genes and liver functions and demonstrated that the dysfunction of circadian rhythm is a driving force of certain liver diseases, such as alcoholic liver disease, liver fibrosis and liver cancer [7]. In the setting of IRI, it has been well known that people who have to work at nighttime have a higher chance of developing cardiovascular disease. Besides, heart attacks happen with greater incidence in the early morning than any other time of the day [8]. Studies using mouse model showed that the heart infarction size is significantly different when heart IRI was induced at different time of the day [9]. This observation indicates that the circadian rhythm could be one of the factors, which affect IRI. This is further confirmed by another study showing that mice with the downregulated circadian gene Per2 exhibited less infarct damage than wild-type mice after IRI in the heart [10]. Apart from light, food is the second essential factor for circadian regulation, it is particularly important for the peripheral clock [11]. It can be inferred that feeding behavior also plays a role in the recovery after surgeries. To our knowledge, there are still no investigations

focusing on the interaction between circadian genes, food intake and hepatic IRI. Therefore, we aim to clarify the role of circadian rhythm in the outcome of experimental model of hepatic ischemia and reperfusion injury..

Materials and methods

Animals

All animal experiments were approved by Cantonal Veterinary office of Zurich and were performed according to Swiss Federal Animal Regulations. Male wild-type mice (C57BL/6, Company) aged 10-12 weeks were used in all the experiments. At least 3 weeks prior to experiments, mice were housed under strictly controlled conditions in a 12-hr light/12-hr dark cycle to entrain the circadian clock. All animals received laboratory chow and water ad libitum.

Surgical procedure and time points

Under isoflurane/O2 inhalation anesthesia, animals were subjected to laparotomy and 70% segmental ischemia as previously described [12]. The blood inflow to the median and left lobes of the liver was interrupted by a micro vascular clamp for 60 min. Reperfusion followed by removing the clamp. Surgeries were performed on mice at ZT0, ZT7, ZT12, ZT16 or ZT18 (ZT, Zeitgeber time in a 12-hr light/12-hr dark cycle; ZT0 represents lights on and ZT12 represents lights off) to compare differences of hepatic IRI. For those animals who were subjected to fasting, fasting started 6h before the induction of ischemia at ZT0 and ZT11.

Melatonin treatment

Physiological dose (0.9 ng/ml) of melatonin (MLT) was given intravenously shortly before the operation at ZT7 and ZT18.

Serum transaminase

Blood sample obtained from the inferior vena cava was centrifuged immediately at 6000 rpm for 6 min, supernatant was acquired for analysis. Aspartate aminotransferase (AST), alanine aminotransferase (ALT) were measured by a serum multiple biochemical analyzer (Dri-Chem 4000i, Fujifilm).

Enzyme Linked Immunosorbent Assay

Serum HMGB1 level was measured by enzyme linked immunosorbent assays (Shino Test). The procedure was based on the instructions, triplicate measurements were performed.

Quantitative real-time PCR

Total RNA was extracted from 50 mg of liver tissue using Trizol reagent (Invitrogen, Basel, Switzerland). RNA quality/quantity was checked by a spectrophotometer and then converted to cDNA. qPCR was performed using an ABI Prism 7500 Sequence Detector system (PE Applied Biosystems Rotkreuz, Switzerland). TaqMan gene expression assays for IL-6 (Mm00446190_m1), TNF-a (Mm00443258_m1), Per1 (Mm00501813_m1), Per2 (Mm00478099_m1), Bmal1 (Mm00500226_m1), Cry1 (Mm00514392_m1), Cry2 (Mm01331539_m1) and 18S rRNA internal control were purchased from Applied Biosystems. Results represent mean fold induction ($2^{-\Delta Ct}$) ± SD.

Histological analysis

Histological analysis was done by fixing liver specimens in 4% PBS-buffered formalin and embedding in paraffin. Hematoxylin-eosin (H&E) staining was performed according to instructions.

Immunohistochemistry staining

Immunohistochemisty stainings were performed following protocols. Anti-HMGB1 antibody (Abcam, 18256) was used as primary antibody. Ventana Discovery automated staining system and the iView DAB kit (Ventana Medical Systems, Tucson, AZ) were used as secondary detection. The staining was done by Sophistolab AG (Muttenz, Switzerland) according to provided instructions.

Statistical analysis

Data are expressed as mean ± standard deviation. Differences between groups were evaluated by an unpaired t test, At least 6 mice/group were analyzed. Statistical analysis was performed by Prism 6.0 (GraphPad) and differences were considered significant at $P < 0.05$.

Results

Dose hepatic ischemic reperfusion injury differ when operating at different time of the day

To evaluate whether circadian rhythm has an influence on hepatic IRI, mice were divided into different groups. Surgery was performed at ZT0, ZT7, ZT12, ZT16 or ZT18. ZT0-ZT12 represents daytime and ZT12-ZT0

represents nighttime. Serum and tissue samples were harvested at 6h after reperfusion in all time points and at 24h after reperfusion in ZT7 and ZT18 groups. After 6h of reperfusion, the mice undegoing surgey at nighttime exhibited lower level of AST and ALT compared with those operated during daytime (Fig 1. A). The difference between ZT7 and ZT18 was statistically significant (Fig 1. B). After 24h of reperfusion, the difference of AST and ALT levels between ZT7 and ZT18 groups disappeared (Fig 1. C). These results indicated that the time of operation has an effect on hepatic IRI. Surgery at nighttime showed less injury to the liver after 6h of reperfusion compared to surgery at daytime. This difference disappeared after 24h of reperfusion.

High-mobility group box 1 (HMGB1) is an inflammatory cytokine that mediates hepatic IRI in the early phase. It translocates from the nucleus to the cytoplasm and is released into circulation when tissue damage occurs. Thus it can be used as a marker of tissue injury. Hepatic IRI at different time points was further measured by circulating HMGB1. When operations were performed at nighttime, serum HMGB1 levels were lower than after daytime operations ($p<0.05$) (Fig 1. D). This was confirmed by immunohistochemistry showing less HMGB1 translocation from the nucleus to the cytoplasm when operating at ZT18, compared with ZT7 (Fig 1. E). The change of HMGB1 levels was consistent with the change of AST and ALT and further demonstrated that operation time has an influence on hepatic IRI.

Dose hepatic IRI change the expression of circadian genes?

The circadian gene expression in the peripheral organs, such as liver, could be affected by either central clock from suprachiasmatic nucleus or external stress [13]. Surgery is a kind of stress which may change circadian gene expression patterns and influence the behavior of the animals. Therefore, we assessed the expression of different major circadian genes in order to find out the correlation between circadian gene expression and hepatic IRI at different time points. Among hundreds of circadian genes, Per1, Per2, Bmal1, Cry1, Cry2 are considered the dominant genes which form the circadian loop and control the expression of other circadian genes. In our study, the expression profile of each circadian gene was shifted under the stress of hepatic IRI (Fig 2). It indicates that surgical intervention affects the expression of circadian genes. This shift may also result in the difference of hepatic IRI when operating at different time points of the day.

Does endogenous MLT influence the outcome of hepatic IRI when operating at different time points?

MLT is a hormone mainly produced by the pineal gland. It is involved in the entrainment of the circadian rhythm. The secretion of MLT is also circadian based, which reaches peak acivity at nighttime and returns to baseline during daytime. Therefore, it regulates sleep-wake timing. In our animal model, C57BL/6 mice do not

have significant amounts of MLT, with little fluctuation between daytime and nighttime [14, 15]. Thus, we injected a physiological dose of MLT to those animals at ZT7 and ZT18 to see whether endogenous MLT influences hepatic IRI between daytime and nighttime operations. With physiological MLT treatment, we did not observe any difference in AST and ALT levels at both ZT7 and ZT18 compared with non-treated group (Fig 3. A). Furthermore, MLT injection did not affect the expression of each circadian gene under the stress of IRI (Fig 3. B). It showed that the difference of hepatic IRI at ZT7 and ZT18 is independent of MLT .

Dose fasting play a role in hepatic ischemic reperfusion injury when operating at different time of the day

Although the circadian rhythm is mainly entrained by light/dark cycles, feeding behavior also plays a big role in this rhythm, especially for peripheral organs [16]. Food intake should not be ignored when investigating the influence of the circadian rhythm. Therefore, besides light/dark cycle, we also took food into consideration in our study. Based on our observation that a significant difference in hepatic IRI could be observed between ZT7 and ZT18, we selected those two time points for the fasting experiment. To mimic the clinical situation, 6h fasting was applied to some animals in the two time points. Interestingly, we observed completely different effects of fasting between operations at daytime and nighttime. During daytime, 6h fasting significantly attenuated hepatic IRI (Fig 4. A), whereas, fasting at nighttime worsened hepatic IRI injury compared with non-fasting group (Fig 4. B). Furthermore, the protective effect of nighttime operation disappeared when mice were subjected to 6h fasting. We even observed a significant increase of AST levels after nighttime operation compared with daytime operation. Thus, the protective effect of nighttime operation was reversed when the animals were fasted. These results indicated that fasting seems to be protective for daytime surgeries and harmful for nighttime surgeries and demonstrated that besides light, food plays an essential role in circadian regulation.

Discussion

Due to the flexibility and unpredictable availability of donor livers for transplantation, surgery could take place at any time of the day. In this study, we aim to elucidate whether the operation time has an impact on hepatic injury and patients' outcomes. We report our experimental findings that hepatic IRI is different when operating at different time points of the day. Nighttime operations showed a significant protective effect compared with daytime operations. Besides, we demonstrated an impact of food intake on hepatic IRI injury by observing that 6h fasting could abrogate the protective effect of nighttime operations.

Circadian rhythm is involved in various physiological activities and plays important roles in different diseases. A good understanding of circadian rhythm and its working mechanism in the human body will help us understand better human health. Surgery has been paramount for medical progress. However, unlike most surgery, , liver transplantation has to be performed as soon as possible, independent of time of day. Its timing depends primarily on the arrival of the donor livers and delay of operation will impair graft quality and outcome of transplantation. The circadian rhythm affects many physiological processes, especially in cardiovascular diseases. The high incidence of heart attack during nighttime indicates that the circadian rhythm plays a role in organ injury [8]. This phenomenon can also be projected to other organs. Indeed, previous studies demonstrated that the liver has the highest number of active circadian genes [17]. Therefore, its function is more likely to be influenced by circadian genes, which is underlined by several reports showing circadian gene dependent liver diseases [7]. Therefore, we hypothesize that the circadian genes are also affecting injury of the liver after IR.

Based on the circadian landscape of the cistrome and epigenome in the liver [18], we selected five time points that represent the peaks of different circadian gene activities. ZT7 represents daytime, ZT16 and ZT18 represent nighttime, ZT0 and ZT12 represent the transition phase between day and night. Nighttime operations exhibit less hepatic IRI compared with daytime operations. The IRI were highest when operations were performed at ZT7, a significant protective effect was seen when operations performed at ZT18. This seems different from the report of human data that showed night surgeries are related to higher risk of early death [1]. However, that the circadian rhythm, although light-dependent, reflects the sleep/wake activity. Since mice are nocturnal animals, preferentially sleep during the day and are active during night [19]. Thus, they have a shifted circadian activity pattern compared to human beings. Our data also show that the beneficial effect of nighttime operations appeared at an early stage while no difference in hepatic injury was observed after 24h of reperfusion . Our results, together with previously reported human data indicate that surgeries at the active phase (daytime for human and nighttime for mice) might be beneficial.

Surgical interventions can disturb sleep and wake rhythm and represent a major problem for patients' outcome [20, 21]. It is correlated with various postoperative complications and diseases [22-24]. What's more, circadian disturbances become more pronounced with liver surgery because the liver exhibits the highest number of active circadian genes [17]. The shift of expression of each circadian gene in our study demonstrated that liver surgery could affect circadian rhythm through influencing circadian genes. However, our current e results cannot provide sufficient evidence to identify which circadian gene is playing a key role in the poor postoperative outcome.

Apart from circadian genes, the endocrine system and hormone levels are actively involved in the regulation of circadian rhythm [25]. Among those hormones, MLT is regarded as the most important for the regulation of circadian rhythm. MLT stabilizes BMAL1 [26, 27] and promotes sleep through MLT receptor 2 (MT2) [28]. The pineal gland of the mouse strain that we have used in this study does not produce MLT because the enzymatic activities for the synthesis of MLT are reduced [29]. Therefore, C57BL/6 mice do not exhibit a MLT peak at night, in contrast with other strains. Hence, C57BL/6 mice are ideal to study the impact of circadian genes on hepatic IRI without the influence of MLT . However, this strain cannot reflect the physiological processes of other animals and humanswho have distinct circadian changes in MLT levels between day and night. Thus, we injected the physiological amount of MLT into circulation before surgery. This physiological peak of MLT level didn't affect the outcome and circadian genes expression of the animals who were subjected to hepatic IRI at both ZT7 and ZT18. Previous studies have shown that high doses of MLT reduced liver damage after warm IRI. This protective effect results from the anti-oxidative property of MLT as well as several other mechanisms [30, 31]. In this study, we demonstrated that physiological doses of MLT do not demonstrate the same beneficial effect as high doses. In addition, the protective effect of a nighttime operation is MLT independent.

In addition to light, food is also a powerful entrainer of circadian rhythm. The impact of food is particularly important because short time fasting before surgery is routine for abdominal operations. The liver plays a crucial role in glucose, lipid, and protein metabolism; it also serves as the first station for dietary carbohydrate, amino acids and nutrients from the hepatic portal vein [32]. Our group has previously demonstrated that 24 hour fasting is beneficial for hepatic warm IRI [9]. When taking circadian rhythm into consideration, more than 12 hour fasting may alter the circadian rhythm and metabolism [33]. Thus, we used 6 hour as short-term fasting time, also because 6h fasting starts always at the beginning of daytime and nighttime, which means daytime fasting wouldn't affect the eating behavior during nighttime and vice versa. Interestingly, we observed different effects of fasting between daytime and nighttime operations. The protective effect of nighttime surgery was lost with 6h fasting while fasting showed beneficial effect on daytime operations. As nocturnal animals, mice have completely opposite eating habits compared to humans. They consume 80% of food intake during the dark phase and only 20% duringthe light phase [34]. It indicates that food deprivation at the time when mice are active is more harmful for hepatic IRI in the early phase. This finding is consistent with the theory that peripheral clocks are predominantly responsive to feeding behavior and the liver clock responds to feeding activity particularly rapidly. When food is restricted for a period of several hours, adverse metabolic consequences and chronic disease may occur [35]. However, we have to always be careful when translating the data to humans, because the

circadian rhythm between human and mice are different, further evidence is needed to evaluate whether the same is true in humans.

In summary, we show that (i) nighttime surgery attenuated hepatic IRI compare with daytime surgery; (ii) surgery of hepatic IR changes the expression of dominant circadian genes at different time points; (iii) The difference of hepatic IRI when operating at daytime or nighttime is MLT level independent; and (iv) short-term fasting is protective for daytime operations and harmful for nighttime operations in terms of hepatic IRI. Altogether, our observations raise an interesting point concerning timing of liver surgeries. The influence of light and food is actively involved in the early recovery after hepatic operations. More investigations on detailed mechanisms and human data are still needed.

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Figure legends

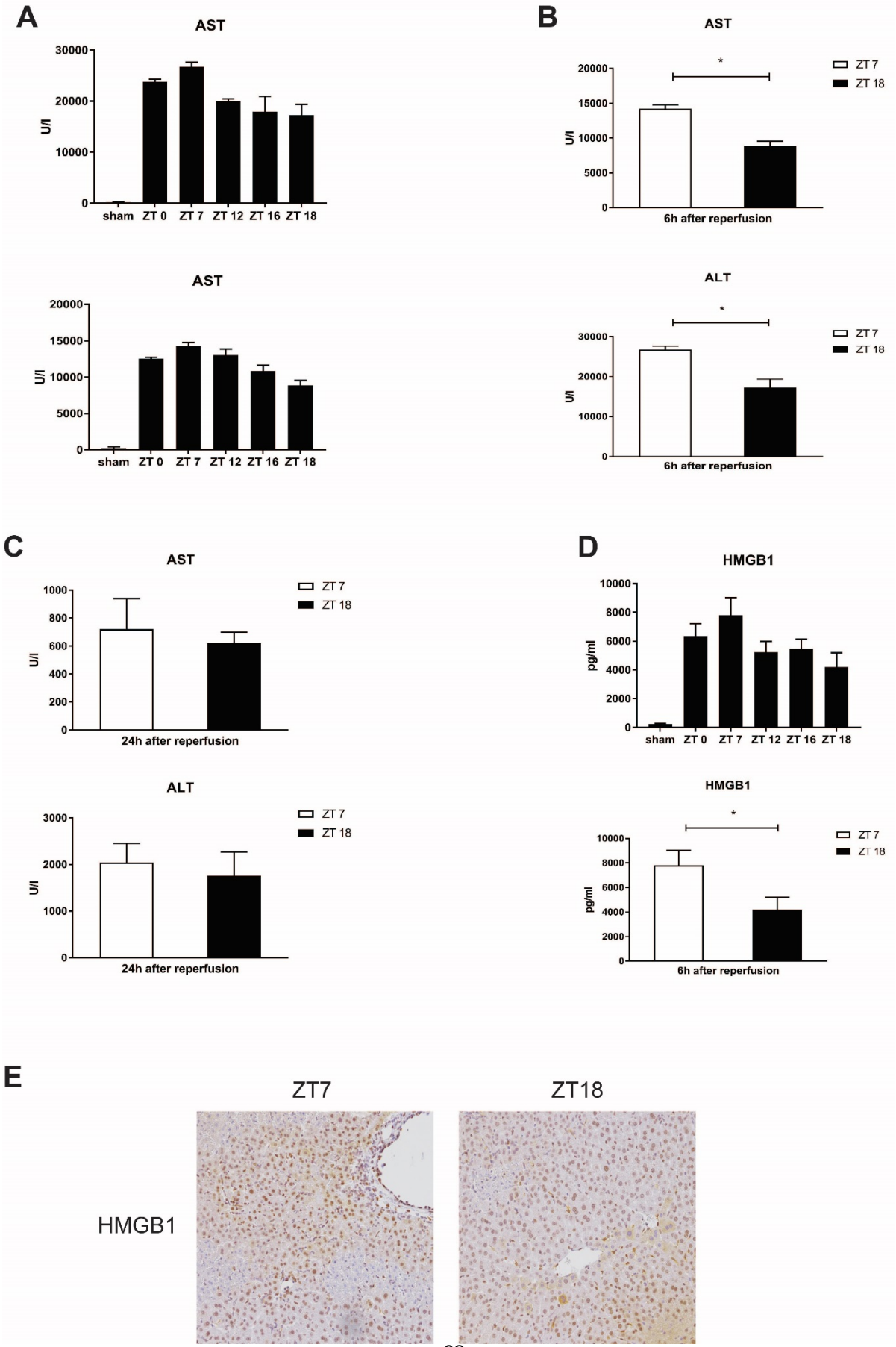
FIG. 1. Markers of hepatic warm IRI when operating at different times of the day. (A) Serum level of AST and ALT after 6h of reperfusion at different time points (n=5 in each group). (B) Comparison of hepatic IRI markers after 6h of reperfusion when operating at ZT7 and ZT18 (n=5). (C) Comparison of hepatic IRI markers after 24h of reperfusion when operating at ZT7 and ZT18 (n=5). (D) ELISA analysis of circulating HMGB1 at different time points (n=5) with a specific comparison between ZT7 and ZT18 (n=5). (E) Immunohistochemistry of HMGB1 (20×) 6 hours after reperfusion when operations were performed at ZT7 (n=5) and ZT18 (n=6). Data are shown as mean ± standard error. *p<0.05.

FIG. 2. Expression shift of dominant circadian genes after 6h of reperfusion when operating at different time points. qPCR on liver RNA was performed and given as fold induction (n=5).). Data are shown as mean ± standard error. *p<0.05.

FIG. 3. The impact of endogenous MLT on (A) hepatic IRI and (B) circadian gene expression at ZT7 and ZT18 (n=5). Data are shown as mean ± standard error. *p<0.05.

FIG. 4. The influence of short-term (6h) fasting on hepatic IRI after 6h reperfusion when operating at (A) ZT7 and (B) ZT18 (n=5). Data are shown as mean ± standard error. *p<0.05.

Figure 1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ZT 18
pg/ml
6h after reperfusion
E
ZT7
ZT18
HMGB1

Figure 2

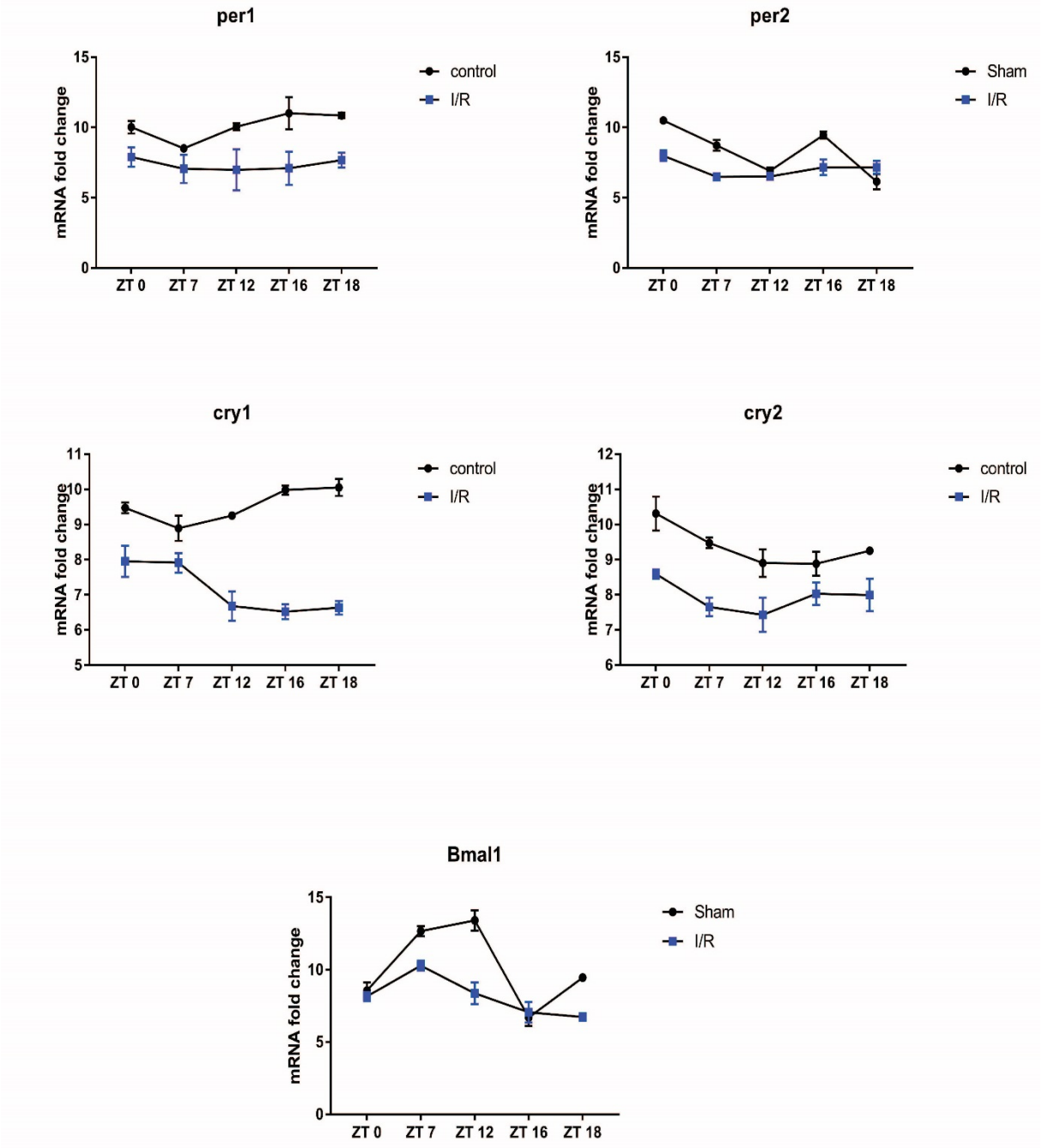
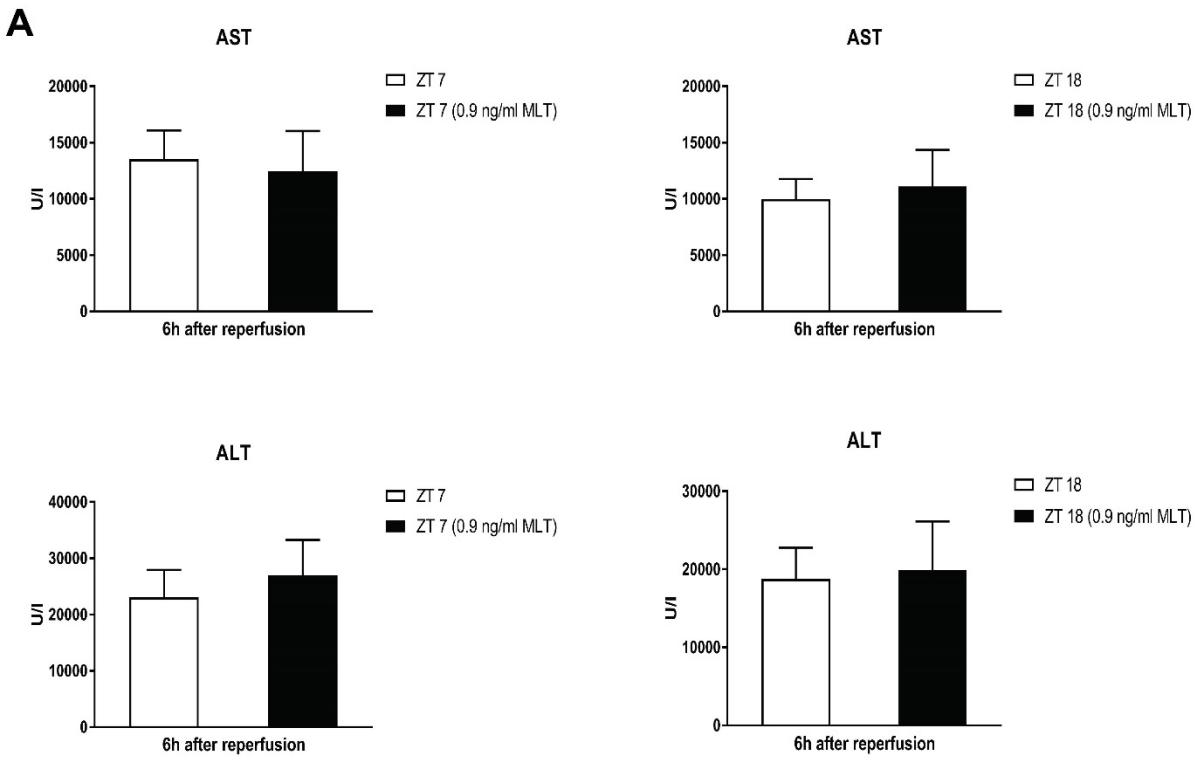

per1
mRNA fold change
15
10
5
0
ZT 0
ZT 7
ZT 12
ZT 16
ZT 18
control
I/R
per2
mRNA fold change
15
10
5
0
ZT 0
ZT 7
ZT 12
ZT 16
ZT 18
Sham
I/R
cry1
mRNA fold change
11
10
9
8
7
6
5
ZT 0
ZT 7
ZT 12
ZT 16
ZT 18
control
I/R
cry2
mRNA fold change
12
11
10
9
8
7
6
ZT 0
ZT 7
ZT 12
ZT 16
ZT 18
control
I/R
Bmal1
mRNA fold change
15
10
5
0
ZT 0
ZT 7
ZT 12
ZT 16
ZT 18
Sham
I/R

Figure 3


A
AST
ZT 7
ZT 7 (0.9 ng/ml MLT)
U/l
20000
15000
10000
5000
0
6h after reperfusion
AST
ZT 18
ZT 18 (0.9 ng/ml MLT)
U/l
20000
15000
10000
5000
0
6h after reperfusion
ALT
ZT 7
ZT 7 (0.9 ng/ml MLT)
U/l
40000
30000
20000
10000
0
6h after reperfusion
ALT
ZT 18
ZT 18 (0.9 ng/ml MLT)
U/l
30000
20000
10000
0
6h after reperfusion

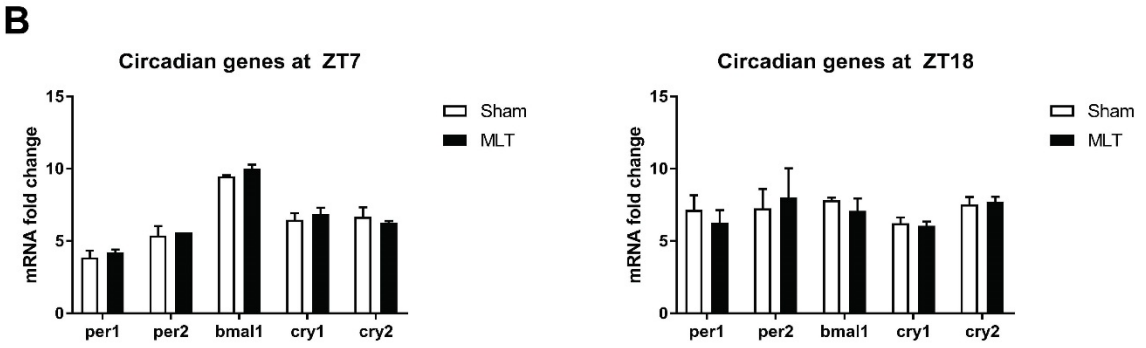
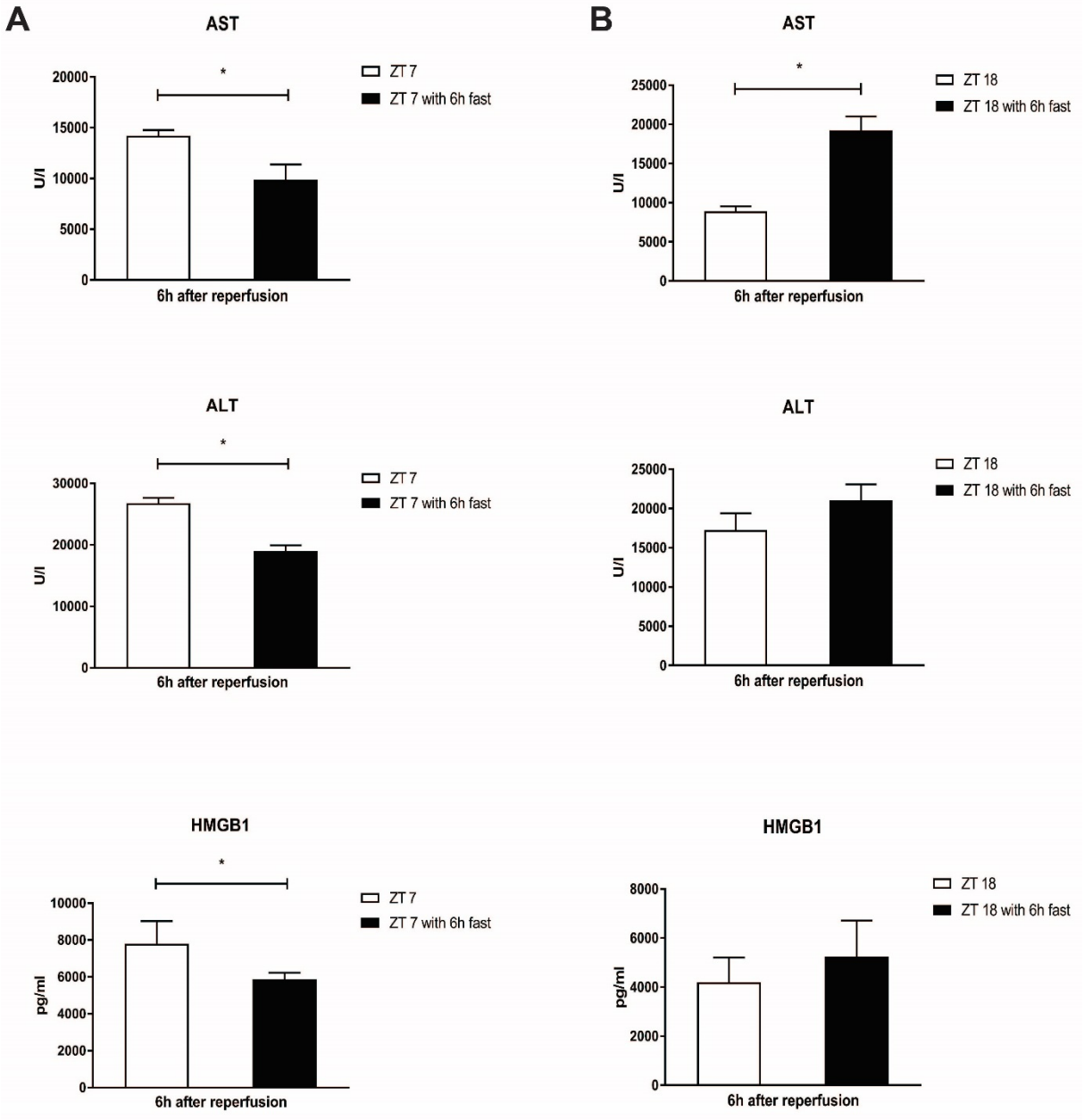

B
Circadian genes at ZT7
Sham
MLT
mRNA fold change
15
10
5
0
per1
per2
bmal1
cry1
cry2
Circadian genes at ZT18
Sham
MLT
mRNA fold change
15
10
5
0
per1
per2
bmal1
cry1
cry2

Figure 4


A
AST
*
20000
15000
10000
5000
0
U/l
6h after reperfusion
ZT 7
ZT 7 with 6h fast
B
AST
*
25000
20000
15000
10000
5000
0
U/l
6h after reperfusion
ZT 18
ZT 18 with 6h fast
ALT
*
30000
20000
10000
0
U/l
6h after reperfusion
ZT 7
ZT 7 with 6h fast
ALT
25000
20000
15000
10000
5000
0
U/l
6h after reperfusion
ZT 18
ZT 18 with 6h fast
HMGB1
*
10000
8000
6000
4000
2000
0
pg/ml
6h after reperfusion
ZT 7
ZT 7 with 6h fast
HMGB1
8000
6000
4000
2000
0
pg/ml
6h after reperfusion
ZT 18
ZT 18 with 6h fast

7. Discussion

7.1 MLT reverses SFS liver graft failure

An effective strategy to prevent or treat SFSS is essential to expand the application of SFS liver grafts in LDLT, and further increase the liver donor source. In this study, we revealed that exogenous MLT is a powerful agent in preventing SFSS after SFS liver transplantation in mice. We demonstrated that MLT reduces hepatic IRI and promotes graft regeneration through activating IL-6/gp130-Stat3 pathway. Furthermore, MLT shows a dramatic protective effect on the microcirculation of small liver graft.

In liver transplantation, IRI is caused by the absence of oxygen during ischemia, and the oxidative stress brought by the blood after the reperfusion.,. Activation of innate immunity, release of cytokines and chemokines, and a series of immunological cascades are considered as the underlying mechanisms of IRI [126]. Among these mechanisms, the generation of reactive oxygen species (ROS) by mitochondrial during the reperfusion phase is the typical manifestation of oxidative stress. The exceptional anti-oxidative effect of high dose MLT has been demonstrated in different organs, especially in hepatic IRI animal models. To investigate the role of MLT in small liver graft transplantation, we firstly used I/R+PH model to simulate SFS liver transplantation and confirmed the previous reports that only a high exogenously dose (10 mg/kg) of MLT is effective. With this dosage, we observed a significant hepatocyte protective effect of MLT in terms of reduced AST and ALT levels from 16 to 48 hours after I/R+PH, this was further supported by *in vitro* experiment that MLT inhibited the release of AST and ALT from primary mouse hepatocyte into the cell culture medium.

HMGB1 is an ancient nuclear protein which plays a crucial role in transcription regulation, it bears a highly conserved sequence and is widely expressed by many cell types [127]. It is well known that HMGB1 is a danger signal because the plasma HMGB1 level could significantly elevate within 1 hour after injury and stress [128]. HMGB1 is also regarded as an essential factor during oxidative stress [129]. In the circulation of our I/R+PH model, we observed two peaks of HMGB1 level in MLT free mice, at 1 and 24 hours respectively. In the MLT treated mice, only one peak at 1 and 3 hours post-I/R+PH was seen. There are two types of circulatory release of HMGB1, it can be actively secreted by immune cells or be passively released by necrotic cells [130, 131]. Our data indicated that the first HMGB1 peak in both groups is most likely attributed to immune cell secretion, MLT didn't inhibit this process because the early immune response is important in cell protection and proliferation after injury [132]. However, MLT dramatically reduced the second HMGB1 peak from 16 to 48 hours post-I/R+PH, this result is consistent with the findings of AST and ALT levels. Thus, we can draw the conclusion that

the second peak of HMGB1 is released from necrotic hepatocyte, and MLT treatment significantly reduced the damage to hepatocyte. In addition, we observed a massive translocation of HMGB1 from the nucleus into the cytoplasm with immunostaining 24 hours post-I/R+PH. HMGB1 translocation is a classic response after hepatic IRI, it has to translocate into the cytoplasm before releasing into the circulation from the cells that have lost the integrity of membrane. The cytoplasm translocation of HMGB1 at 24 hours is in accordance with its circulatory release, and this kind of translocation was also significantly inhibited by MLT treatment.

A normoxic range of oxygen concentration is important to maintain normal cellular function, a gradient level of oxygen tension in all mammals is the first defense system of the toxicity of oxygen, this system keeps a low tissue levels in order to prevent oxidative damage [133]. When the liver is subjected to IRI, mitochondrial generated ROS is a typical product in response to oxidative stress, the excessive production of ROS could destroy the cellular anti-oxidative defense system and lead to oxidative damage to the DNA [134]. Although DNA damage and repair is a normal cellular process, approximately 2×10^4 DNA damage occur in each cell of the human per day [135]. ROS has the ability to damage DNA bases and cause DNA double strand break (DSB) which is a lethal process through inactivating key genes and chromosomal aberrations [133]. In order to evaluate the role of MLT in DNA damage protection after I/R+PH, we performed immunostaining of Gamma-H2A.X which phosphorylates rapidly at DSB sites and further recruits damage response proteins. 3 hours after I/R+PH, we observed scattered distribution of Gamma-H2A.X positive cells in vehicle treated mice, indicating DSB occurred at an early stage post-I/R+PH, even before the release of liver enzymes. In MLT treated mice, the positive cells are scarcely seen in the liver tissue. This result demonstrated that MLT treatment could protect the liver in the DNA level after I/R+PH.

Previous studies using warm hepatic IRI murine model demonstrated that the hepatocyte protective effect of MLT [90] accompanied by the reduced level of IL-6, the beneficial effect was attributed to anti-inflammatory effect because IL-6 is considered as pro-inflammatory cytokine. In contrast, the murine model of 70% hepatectomy elevates the level of IL-6 at the early phase after surgery, results in robust liver regeneration. It indicates IL-6 is essential for liver regeneration and hepatic protection and IL-6 has anti-inflammatory property. Thus, IL-6 exerts equal hepatic protective effects in both IRI and PH models by opposite expressions. However, when we merge IRI and PH models into one I/R+PH model, MLT increased IL-6 level shortly after the early phase and delayed anti-inflammatory activities, which combined both results of MLT treated IRI and PH. Besides its role in inflammation, IL-6 and TNF-α are indispensable for liver regeneration and hepatocyte protection [136, 137]. IL-6 is not only a pro-inflammatory factor, but also has context-dependent anti-inflammatory

properties [138]. Hence, we speculate that the elevation of IL-6 and TNF-α level plays a vital role in liver regeneration and hepatocyte protection after I/R+PH. Immunostaining of PCNA and pH3 as well as the liver to body ratio results 2 days after surgery confirmed our speculation.

With the encouraging data in I/R+PH model, we further tested the effect of MLT in 30% arterialized partial liver transplantation model, this is a well established model of small for size liver transplantation (SFS-LT) with almost 0% animal survival rate after 7 days of surgery [139]. However, MLT treatment exhibited its hepatic protective and regenerative effect by achieving more than half of the animals survived more than 7 days. In the cytokine level, MLT elevated the mRNA expression of IL-6, TNF-α and HGF level 6 hours post-SFS-LT, in particular, the early significant elevation of IL-6 at 3 hours post-SFS-LT which is earlier than the other cytokines drew our attention to further investigate the mechanism of MLT through IL-6 signaling. On the protein level of IL-6 downstream pathway, we noticed an upregulation of gp130 expression and STAT3 phosphorylation, which are crucial components of IL-6 signaling after 3 h of transplantation [140], demonstrating the essential role of IL-6 pathway in the beneficial effect of MLT. In the functional study, we confirmed that the protective effect of MLT in terms of animal survival disappeared with the blockage of gp130. Furthermore, the enhanced liver regenerative capability vanished in IL-6 knock-out mice even with the injection of MLT, and the effect of MLT was restored by rIL-6 treatment. All these findings supported that IL-6-gp130/stat3 pathway is indispensable for the beneficial effect of MLT in SFS liver transplantation settings. Although the role of IL-6 in liver regeneration remains controversial, we still proved its efficacy in our study. In extended hepatectomy and SFS liver transplantation, the small remnant liver or graft requires stronger regenerative response to restore the tissue loss [141], therefore, the early phase elevation of IL-6 in our study can be explained as an essential response to trigger the regeneration of liver graft after SFS liver transplantation. In the model of hepatic IRI, the liver was only subjected to warm IRI without hepatectomy, acute phase cytokines are not necessary to trigger a massive regenerative response. That is also the reason why previous studies reported the decreased IL-6 level after MLT treatment in the pure IRI model. These results indicate a dual regulative function of MLT, it can upregulate or downregulate the level of cytokines based on the needs of the human body. Apart from its pro-regenerative role, Blindenbacher et al [142] reported that IL-6 is important for animal survival after partial hepatectomy, and the elevated level of IL-6 showed hepatocyte protective effect. Hence, the increase of IL-6 level is not just a sign of inflammation, but also a positive factor in hepatocyte protection and the initiation of liver regeneration. The effect of MLT on IL-6 signaling in our study supports strongly this conception.

The hepatic IRI together with the injury of surgical manipulations are associated with the damage of microcirculation and increased capillary permeability in the liver [143]. Leukocyte activation is the cellular response for IRI, which is presented by rolling and sticking of leukocytes in sinusoids and post-sinusoidal venules [144]. In our I/R+exPH model, the small remnant liver displayed significantly decrease of sinusoid perfusion and increase of leukocyte adhesion, indicating a severe impairment of hepatic microcirculation. The treatment of MLT reversed this damage and retained almost intact microcirculation as sham animals. The release of nitric oxide (NO) is related to hepatic IRI, there are two isoforms of NOS: endothelial NOS (eNOS) and inducible NO synthase (iNOS). eNOS is expressed in sinusoidal endothelial cells and is crucial in protecting the vascular endothelium, its upregulation triggers the protective effect of endothelium through increasing blood flow [145]. In contrast, iNOS is expressed in all liver cells and promotes the formation of free radical, thus, iNOS is considered as an IRI promotor [146]. A previous study has reported that MLT could upregulate the expression of eNOS and downregulate the expression of iNOS. This feature suggests that MLT could improve hepatic microcirculation through sinusoidal vasodilation and blood flow increase [147]. In addition, after SFS liver transplantation, the elevated portal pressure and reduced hepatic arterial perfusion lead to endothelial damage, the endothelial dysfunction together with ROS accumulation worsens hepatic microcirculation and may eventually cause liver failure. Therefore, hepatic microcirculation protection is particularly important in functional recovery and hepatocyte proliferation for SFS liver graft. Our study proved that the protective effect of MLT in hepatic microcirculation is associated with better hepatic function and regeneration.

In conclusion, we demonstrated for the first time that MLT is a potential beneficial agent in SFS liver transplantation, the protective role displays as the improvement of hepatic IRI and the enhancement of liver regeneration. This effect attributed to the activation of IL-6/gp130 dependent Stat3 pathway and the preservation of SFS graft's microcirculation.

7.2 Circadian rhythm affects hepatic ischemia reperfusion injury in mice

In order to answer the question whether operating time matters in hepatic IRI, we performed 70% hepatic IRI in mice at different time points of the day, we found that hepatic IRI varies when operating at different time and nighttime operations showed the most significant protective effect. In addition, fasting at daytime and nighttime exhibits different effects on hepatic IRI.

Circadian rhythm is one of the most important regulatory systems of the human body, it participated in a variety of body functions, the disturbance of circadian rhythm will cause the dysfunction of organs and lead to diseases [114]. Surgical interventions disturb the circadian rhythm and affect the patients' outcome [148], this phenomenon is more pronounced with liver surgeries since the liver has the most circadian genes among all the organs [115]. Our study confirmed this theory by observing a dramatic shift of different circadian gene expression in the liver 6 hours after surgery, the shift is associated with different outcomes when operating at different time points after hepatic IRI. The time points we selected in our study is based on the circadian landscape of the cistrome and epigenome in the liver [149]. These points can well represent the peak of different circadian gene expression and also fit the clinical situations of liver transplantation. A previous study has already demonstrated that operating time influences the regenerative capability after hepatectomy [150], therefore, in this study we just focus on the impact of circadian rhythm in hepatic IRI, which, to our knowledge, has not been elucidated. We noticed that nighttime operation exhibited significant protective role in decreasing hepatic IRI 6 hours after surgery, and this effect vanished with 6 hours fasting before the surgery. However, 6 hours fasting in the daytime operated mice showed reduced hepatic IRI compared with non-fasting animals which is completely different from the nighttime operated mice. These data has inspired us to pay more attention to the peri-operative management of liver transplant surgery because both timing and fasting play roles in the short-term outcomes of experimental animals subjected to hepatic IRI. Whereas, to correctly interpret the results, we need to bear in mind that mice are nocturnal animals, which means they have totally opposite daily behavior and eating habit with human beings [151]. Nights are the active phase for mice and they eat more than 80% of food, what we observed was an attenuated liver injury when operating during the active phase of mice, and food restriction is harmful. To translate into humans, it provides an information that a better outcome might be achieved when liver surgeries are performed during the active phase which is during the daytime, and whether fasting is necessary or what is the appropriate fasting time when operations have to be done at different time of the day still needs to be further studied. With this observation, we opened a window to look at the impact of circadian rhythm on hepatic IRI, however, more research are needed to investigate the detailed mechanisms and to test whether what we have observed in mice can be extrapolated into humans.

In addition, this study provides us a good animal model to replenish our main project concerning the role of MLT in SFS liver transplantation. It is well accepted that MLT is a circadian regulator which exhibits daily fluctuation of secretion [76]. In our main project, we observed significant protective effect of exogenous high dose MLT in protecting the function and promoting the regeneration of small liver graft. In order to further investigate the mechanism of MLT, we applied hepatic IRI model to test whether its physiological circadian regulative

effect also affects animal outcomes. Due to the compromised enzymatic activities, the pineal gland of C57BL/6 mice do not have the capability to produce MLT [152], although it owns MLT receptors. Therefore, we injected endogenous dose of MLT to mimic the physiological peak of MLT at ZT7 and ZT18. However, on the contrary of high dose MLT, the physiological peak of MLT didn't show any protective effect in liver injury after hepatic IRI. We have previously demonstrated that the blockage of MLT receptors didn't eliminate the beneficial role of high dose MLT in our I/R+PH model, meaning the effect of MLT is receptor independent, because the lipophilic feature of MLT enable it to enter the cell without binding to its receptors. Thus, the role of MLT in circadian regulation is dependent on its receptors while the high dose MLT is more prone to function in the receptor independent way [78]. Our finding provides a proof that the hepatic protective role of high dose MLT is independent of MLT receptors, and confirmed that physiological MLT secretion does not act a role in its beneficial effect in small liver grafts.

7.3 Clinical relevance and future perspectives

In this thesis, we reported two clinical relevant investigations, one study revealed a new strategy to prevent SFSS after SFS liver transplantation, the other one provides a new viewpoint that circadian rhythm influences short-term outcome after hepatic IRI.

LDLT offers a great idea to increase the source of donor livers, to guarantee the safety of donors and to further increase the availability of SFS liver grafts, it is essential to understand and overcome the SFSS. The currently available strategies of rescuing SFSS are either related to surgical complications or lack of clinical experience. In this study, we found that MLT is a powerful agent to reduce hepatic injury and promote liver regeneration through several protective mechanisms in mouse SFS liver transplantation model. What we observed infers that MLT can be used as a simple, effective and safe treatment to prevent SFSS. In the clinic, MLT has already been applied to treat sleep disorders, and its high dose has been proved safe in liver surgeries. In liver transplantation settings, a recent clinical trial (http://ClinicalTrials.gov/show/NCT01860716) has been designed to investigate the role of MLT in liver donors after brain death. Therefore, the translation of our study into the clinic is feasible and a related clinical trial can be implemented soon. If the efficacy of MLT can also be provided in the clinic, it will offer a new option to prevent SFSS and decrease the limiting threshold of the size of liver graft. By using SFS liver graft, we can potentially increase the source of liver donors and contribute to the solution of organ shortage.

The main difference of liver transplantation from other surgeries is the requirement of speedy organ retrieval and implantation to reduce ischemia time of the donor, thus, the surgery of liver transplantation can be taken place at any time of the day depending on the arrival of donor livers. Our study using hepatic IRI mouse model showed that the short-term outcome of IRI injury is different when the surgery was performed at different time of the day, and fasting during daytime and nighttime exhibits different effect. These results demonstrated that circadian rhythm influences the quality of liver surgery. Although this study is an observation without unveiling the specific mechanisms, still it reminds us operating time of surgery might play a role in the outcome of liver transplantation recipients.

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